administration	for the preparation of standard, oral, intravenous and isotopic diluent solutions. The exact concentrations of these solutions were determined by isotope dilution. The oral solutions contained 0.129 mg ^{65}Cu/g solution in MP1, 0.064 mg/g in MP2 and 0.670 mg/g in MP3. The isotope solutions replaced the natural Cu usually added to the supplement on other days of the study in MP1 and MP3. In MP2, no natural Cu was added to the diet. The ^{65}Cu was fed over 2 days to obtain adequate isotopic enrichment of samples used for absorption determinations with minimum increase in the Cu content of the diet after addition of the isotope in MP2. Polyethylene glycol was fed along with the Cu isotope as a faecal marker. Solutions for i.v. administration were prepared using sterile water for infusion and the pH adjusted to 2.0. Four grams of the solution containing 336 µg ^{65}Cu were infused into arm veins of subjects over 1 minute.
43.4 Examinations	Non-entry field
43.4.1 Body weight	Body weight was monitored throughout the study. The daily energy content of the diet was adjusted as appropriate to ensure that each volunteer would maintain his initial weight.
43.4.2 Faeces collections	Complete faecal collections were made throughout the study. See section 3.5.1 for more information.
43.5 Sample processing and analysis	Non-entry field
43.5.1 Sample collection and processing	Composites of each daily menu were collected 7 times throughout the study for determination of Cu (twice during MP1, three times during MP2 and six times during MP3). Samples of the supplementary drink were saved 6 times during MP1, 9 times during MP2 and 6 times during MP3. Complete faecal collections were made throughout the study and combined into 6-day pools. Diet composites and faecal pools were homogenised, frozen, lyophilised, dried, crushed to a powder and stored.
43.5.2 Preparation of samples for isotope ratio measurements	For each 6-d faecal composite, two 1 g aliquots were weighed into quartz crucibles and a solution containing 10 µg ^{65}Cu was added to one aliquot. After equilibrating overnight, the samples were dried on a hot plate. For every 11 pairs of aliquots weighed in this manner, 2 natural faecal reference composites were weighed. Samples were ashed overnight, treated on a hot plate with a solution of nitric acid, dried, and subjected to a second overnight ashing. Samples were then dissolved in 6 mol ultrapure HCl/litre. Cu was separated and purified by anion-exchange chromatography and samples were concentrated to 0.25 g/l. All diet composites and samples of the supplementary drinks were analyzed in duplicate for Cu. Approximately 2.6 µg ^{65}Cu was added to a 3 g subsample of each composite. Three different amounts of ^{65}Cu were added to the formula composites, depending on the copper content. A rotating menu composite from MP3 was used as a natural diet reference composite. Four subsamples were weighed. After weighing, the samples were ashed, dissolved, purified, and concentrated as the fecal samples.
43.5.3 Isotope ratio determinations	Cu isotope ratios were determined in faecal samples, diet composites, and isotope solutions with a magnetic-sector thermal ionization mass spectrometer. Samples were analysed under computer control, with typical conditions consisting of: evaporation filament current of 1.5 A, filament temperature of 1500 °C. ^{65}Cu ion beam intensity of 300 mV,

analysis time 60 minutes, and single-analysis relative SD of 0.05%.

The ^{65}Cu and total Cu content of the samples were determined by isotope dilution using the 65:63 isotopic ratios of two duplicate aliquots, with and without added ^{65}Cu, unenriched samples, and enriched ^{65}Cu solutions fed, infused, and added as isotopic diluent, along with weights of the sample aliquots and the added isotopic diluent, the concentration of the isotopic diluent, and the total dry weight of the faecal pool or food composite.

43.5.4 Absorption and endogenous excretion calculations

Absorption was calculated as the fraction of fed ^{65}Cu that was not recovered in the stools in the 12 days after the feeding of the oral dose. The recovered ^{65}Cu also included any ^{65}Cu absorbed and then excreted into the gastrointestinal tract during that time. The amount of Cu excreted into the gastrointestinal tract was calculated based on the fraction of the infused dose of ^{65}Cu excreted into the stools and eliminated in the 12 days after the infusion. The average fraction of absorbed Cu that was excreted was then added to the fraction apparently absorbed during each MP, to estimate true absorption during each MP. Slow turnover endogenous losses did not include the absorbed dietary Cu excreted within 12 days. Fast turnover losses, the amount of dietary Cu excreted in 12 days, was based on the 12-day excretion of infused Cu. Total endogenous losses are the total of the two.

43.5.5 Statistical analysis

Statistical analysis was performed with SAS. Descriptive statistics, including means, SDs, and plots, were tabulated and compared. An ANOVA model was used to determine the effects of the 3 dietary Cu intakes on Cu absorption, retention, and excretion. If a significant difference was found, Fisher's least-significant-difference test was used to determine which treatment means differed. The same ANOVA model was used to compare absorption early and late in the copper depletion period. A significance level of 0.05 was used for all statistical tests.

44 RESULTS AND DISCUSSION

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

44.1 Results

Non-entry field

44.1.1 Prestudy dietary copper

The average Cu intake of the subjects in the 5 days before the study began ranged from 0.81 to 2.61 mg/day. Overall average Cu intake was 1.68 ± 0.54 mg/day (mean ± SD).

44.1.2 Copper excretion

The excretion pattern of ^{65}Cu after infusions (12 days before the end of each MP) is shown in **Figure A6.2(08)-1**. This shows that the amount of Cu excreted was lowest when dietary Cu was low and increased with higher intakes of dietary Cu.

The fraction of the infused dose excreted into the gastrointestinal tract in the 12 days after the isotope infusion is shown in **Table A6.2(08)-2**. For MP1, 12% of the dose was excreted in the first 6 days after infusion and another 14% was excreted in the next 6 days. In MP2, 6.9% was excreted in the first 6 days and 5.1% in the next 6 days. In MP3, 20% was excreted in the first 6 days and 14% in the next 6 days. Both 6-day and 12-day excretions were significantly lower with the low-copper diet (MP2) than with the higher copper diet of MP3. The 12-day excretion, but not the 6-day, was significantly lower during MP2 than during MP1.

44.1.3 Copper absorption	Cu absorption during each dietary period is shown in **Table A6.2(08)-2**. One of the 5 subjects included in the absorption study had an extremely slow transit time, making it impossible to calculate absorption. His absorption data were not included. The 67% absorbed during the low-Cu diet was significantly higher than the percentage absorbed during MP1 (54%) and MP3 (44%). The amount of Cu absorbed increased with increasing dietary Cu, averaging 0.26 mg/day during MP2, 0.35 mg/day during MP1, and 1.08 mg/day during MP3. Absorption was measured twice during the low-Cu diet, averaging 71% early in this period and 67% later on. This difference was statistically signoficant. True absorption was estimated by adjusting apparent absorption based on faecal monitoring to account for the amount of Cu absorbed and then excreted during the 12-day collection period. The pattern was similar to apparent absorption, but differences were smaller.
44.1.4 Copper retention (balance)	Average Cu retention for each MP is shown in **Table A6.2(08)-2**. Faecal Cu and Cu retention for 6-day periods throughout the study are shown in **Figures A6.2(08)-2** and **A6.2(08)-3**. The influence of previous dietary Cu intake on faecal Cu can be seen early in each MP. After the first 6-day period, faecal Cu more closely reflected dietary intake. Whereas volunteers were in negative balance early in MP1 and MP2, they were no longer in negative balance by the end of the MPs. Balance was highly positive early in MP3, but declined rapidly after the first 6-day period.
44.1.5 Copper turnover	Endogenous Cu losses, calculated for both slow and fast turnover pools in the final 6 days of each MP, and total endogenous losses via the gastrointestinal tract, are shown in **Table A6.2(08)-3**. When dietary Cu was 0.66 mg/day, 0.13 rng/day of the Cu consumed in the previous 12 days was excreted and eliminated in the stools. When intake was 0.38 mg/day, the amount was only 0.036 mg/day, and when intake increased to 2.49 mg/day, it was 0.55 mg/day. Similarly, endogenous losses not attributed to the previous 12-day intake, or slow-turnover Cu, were influenced by Cu intake. When intake was 0.66 mg/day, 0.34 mg/day was excreted into the gastrointestinal tract and eliminated. When intake was 0.38 mg/day, 0.21 mg/day was eliminated and when intake was 2.49 rng/d, the amount eliminated increased to 0.78 mg/day.
44.2 Discussion	The pre-study dietary Cu intake estimate of 1.68 mgCu/day is generally consistent with the Cu excretion data. Faecal Cu and balance data (**Figures A6.2(08)-2** and **A6.2(08)-3**) show the effect of the higher prestudy Cu intake. Subjects were in negative balance during the first part of the study. In part, the fecal losses reflect elimination of previously consumed dietary Cu and higher endogenous losses due to higher prior intake. Two assumptions were made to estimate absorption: excretion of absorbed dietary Cu and endogenous losses. For absorption calculations, it was assumed that the same fraction of both dietary copper and ^{65}Cu administered orally as an extrinsic label was absorbed. Similarly, for excretion of absorbed Cu and endogenous losses, it was assumed that the same fraction of both absorbed dietary Cu and the infused dose was excreted. This latter assumption is much more speculative and subject to limitations. By infusing the isotope, the normal route of entry into the body is bypassed. The Cu isotope was infused over a short period of time and in a different form than that which enters via the gastrointestinal tract. Despite the differences, the approach was considered sufficiently reliable to roughly estimate endogenous losses and to evaluate the effects of different dietary Cu intakes on endogenous losses.

There appeared to be two points of regulation of body Cu stores. Absorption was more efficient with low dietary Cu intake. This lessened the effect of low dietary Cu, but the amount of Cu absorbed with a low-copper diet was still lower than the amount absorbed when dietary Cu was higher. The second point of regulation, excretion of endogenous Cu into the gastrointestinal tract, may be more important in regulating total body Cu. Isotope tracer data showed (**Table A6.2(08)-2**) that when dietary Cu was lowest, 67% of orally administered ^{65}Cu was absorbed, but only 12% of infused ^{65}Cu was excreted into the gastrointestinal tract. When dietary Cu was highest, 44% of orally administered ^{65}Cu was absorbed and 34% of infused ^{65}Cu was excreted. By using the isotopic tracer, it was possible to separate the endogenous Cu into two components. Some of the absorbed dietary Cu was excreted into the gastrointestinal tract relatively rapidly. That fraction and the absolute amount excreted increased as dietary Cu increased. The change in retention of recently absorbed Cu also aided in regulating body stores by retaining more of the absorbed Cu when intake was low and eliminating the excess Cu absorbed when intake was high. In addition, the remainder of Cu in the body appeared to turn over more rapidly as dietary Cu increased. The decreased rate of turnover of the slow turnover pool when intake was low aided in conserving body Cu stores.

Cu absorption, measured twice during MP2, was significantly higher early in depletion (71%) than late in depletion (67%). The difference was so small that it may not be important physiologically, but it could represent adaptation, in which more Cu was absorbed from the depletion diet early, before endogenous losses declined in response to dietary Cu. This would be consistent with the higher fecal losses observed early in depletion than late in depletion.

Both absorption of dietary Cu and conservation of body Cu work toward adapting to a wide range of dietary Cu intakes. However, at the lowest amount of dietary Cu used in this study (0.38 mg/day in MP2), these adaptive mechanisms were not sufficient for the volunteers to maintain Cu status over the course of the MP.

44.3 Toxic effects, clinical signs

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

No effects reported.

44.4 Recovery of labelled compound

State percentage

Not stated.

45 APPLICANT'S SUMMARY AND CONCLUSION

45.1 Materials and methods

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

A study was conducted in young men to evaluate the effect of a low-Cu diet on Cu absorption, excretion and retention.

Eleven Young men were confined to a metabolic research unit for 90 days. The study was divided into three separate metabolic periods (MP), with dietary Cu as the only variable. Dietary Cu intake was 0.66 mg/day for 24 days (MP1), 0.38 mg/day for 42 days (MP2) and 2.49 mg/day for 24 days (MP3). Dietary Cu was adjusted by the addition of the appropriate amount of a $CuSO_4$ solution to a supplementary drink. The stable isotope ^{65}Cu was fed to 5 of the subjects once during MP1 (days 13 – 14) and MP3 (days 79-80) and twice, early and late, in MP2 (days 31-32

and 55-56) to determine Cu absorption. ^{65}Cu was infused into a vein of the other 6 subjects once during each MP (days 13, 55 and 79 for MP1, MP2 and MP3, respectively) to estimate excretion of endogenous Cu.

Composites of each daily menu and of the supplementary drink were collected at intervals and frozen. Complete faecal collections were also made throughout the study, combined into 6-day pools and frozen. Diet composites and faecal pools were homogenised, re-frozen, lyophilised, dried, crushed to a powder and stored for subsequent determination of Cu.

Two 1 g aliqots were taken from each faecal composite. A solution containing 10 µg ^{65}Cu was added to one aliquot, allowed to equilibrate overnight and dried on a hotplate. Faecal reference composites were similarly treated. Samples were digested in HNO_3, ashed and dissolved in HCl. Cu was separated and purified by anion-exchange chromatography and samples were concentrated to 0.25 g/l. All diet composites and samples of the supplementary drinks were analyzed in duplicate for Cu. Approx. 2.6 µg ^{65}Cu was added to a 3 g subsample of each composite. Different amounts of ^{65}Cu were added to drink composites, depending on Cu content. Reference composites were similarly treated. Four subsamples were weighed. After weighing, the samples were ashed, dissolved, purified, and concentrated as for the fecal samples. Cu isotope ratios were determined in faecal samples, diet composites, and isotope solutions with a magnetic-sector thermal ionization mass spectrometer.

The ^{65}Cu and total Cu content of the samples were determined by isotope dilution using the 65:63 isotopic ratios of 2 duplicate aliquots, with and without added ^{65}Cu, unenriched samples, and enriched ^{65}Cu solutions fed, infused, and added as isotopic diluent, along with weights of the sample aliquots and the added isotopic diluent, the concentration of the isotopic diluent, and the total dry weight of the faecal pool or food composite.

Absorption was calculated as the fraction of fed ^{65}Cu that was not recovered in the stools in the 12 days after the feeding of the oral dose. The recovered ^{65}Cu also included any ^{65}Cu absorbed and then excreted into the gastrointestinal tract during that time. The amount of Cu excreted was calculated based on the fraction of the infused dose of ^{65}Cu excreted into the stools and eliminated in the 12 days after infusion. The average fraction of absorbed Cu that was excreted was then added to the fraction apparently absorbed during each MP, to estimate true absorption during each MP. Slow turnover endogenous losses did not include the absorbed dietary Cu excreted within 12 days. Fast turnover losses, the amount of dietary Cu excreted in 12 days, was based on the 12-day excretion of infused Cu. Total endogenous losses are the total of the two.

ANOVA was used to determine the effects of the 3 dietary Cu intakes on Cu absorption, retention, and excretion. If a significant difference was found, Fisher's least-significant-difference test was used to determine which treatment means differed. ANOVA was used to compare absorption early and late in the copper depletion period. A significance level of 0.05 was used for all statistical tests.

45.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Prestudy dietary Cu: The average Cu intake of the subjects in the 5 days before the study began ranged from 0.81 to 2.61 mg/day. Overall average Cu intake was 1.68 ± 0.54 mg/day (mean ± SD); higher than the intake during MP1. Faecal Cu and balance data show the effect of the higher pre-study Cu intake. Subjects were in negative balance during the first part of the study. In part, the fecal losses reflect elimination of previously

consumed dietary Cu and higher endogenous losses due to higher prior intake.

Cu excretion: The excretion pattern of ^{65}Cu after infusions (12 days before the end of each MP) shows that the amount of Cu excreted was lowest when dietary Cu was low and increased with higher intakes of dietary Cu. For MP1, 12% of the dose was excreted in the first 6 days after infusion and another 14% was excreted in the next 6 days. In MP2, 6.9% was excreted in the first 6 days and 5.1% in the next 6 days. In MP3, 20% was excreted in the first 6 days and 14% in the next 6 days. Both 6-day and 12-day excretions were significantly lower with the lowcopper diet (MP2) than with the higher copper diet of MP3. The 12-day excretion, but not the 6-day, was significantly lower during MP2 than during MPl.

Cu absorption: Cu absorption during each dietary period was assessed. The percentage of Cu absorbed during the low-Cu diet (67%) was significantly higher than during MP1 (54%) or MP3 (44%). The amount of Cu absorbed increased with increasing dietary Cu, averaging 0.26 mg/day during MP2, 0.35 mg/day during MP1, and 1.08 mg/day during MP3. Absorption was measured twice during the low-Cu diet, averaging 71% early in this period and 67% later on. Although statistically significant, this difference was so small that it may not have been important physiologically. It could, however, represent adaptation, in which more Cu was absorbed from the depletion diet early, before endogenous losses declined in response to dietary Cu.

True absorption was estimated by adjusting apparent absorption based on faecal monitoring to account for the amount of Cu absorbed and then excreted during the 12-day collection period. The pattern was similar to apparent absorption, but differences were smaller.

Cu retention (balance): The influence of previous dietary Cu intake on faecal Cu became evident early in each MP. After the first 6-day period, faecal Cu more closely reflected dietary intake. Whereas volunteers were in negative balance early in MP1 and MP2, they were no longer in negative balance by the end of the MPs. Balance was highly positive early in MP3, but declined rapidly after the first 6-day period.

Cu turnover: Endogenous Cu losses, calculated for both slow and fast turnover pools in the final 6 days of each MP, and total endogenous losses via the gastrointestinal tract, were determined. When dietary Cu was 0.66 mg/day, 0.13 rng/day of the Cu consumed in the previous 12 days was excreted and eliminated in the stools. When intake was 0.38 mg/day, the amount was only 0.036 mg/day, and when intake increased to 2.49 mg/day, it was 0.55 mg/day. Similarly, endogenous losses not attributed to the previous 12-day intake, or slow-turnover Cu, were influenced by Cu intake. When intake was 0.66 mg/day, 0.34 mg/day was excreted into the gastrointestinal tract and eliminated. When intake was 0.38 mg/day, 0.21 mg/day was eliminated and when intake was 2.49 rng/d, the amount eliminated increased to 0.78 mg/day.

In conclusion, there were two points of regulation of body Cu stores. Absorption was more efficient with low dietary Cu intake. This lessened the effect of low dietary Cu, but the amount of Cu absorbed with a low-copper diet was still lower than the amount absorbed when dietary Cu was higher. The second point of regulation, excretion of endogenous Cu into the gastrointestinal tract, may have been more important in regulating total body Cu. Isotope tracer data showed that when dietary Cu was lowest, 67% of orally administered ^{65}Cu was absorbed, but only 12% of

	infused ^{65}Cu was excreted. When dietary Cu was highest, 44% of orally administered ^{65}Cu was absorbed and 34% of infused ^{65}Cu was excreted. By using the isotopic tracer, it was possible to separate the endogenous Cu into two components. Some of the absorbed dietary Cu was excreted into the gastrointestinal tract relatively rapidly. That fraction and the absolute amount excreted increased as dietary Cu increased. The change in retention of recently absorbed Cu also aided in regulating body stores by retaining more of the absorbed Cu when intake was low and eliminating the excess Cu absorbed when intake was high. In addition, the remainder of Cu in the body appeared to turn over more rapidly as dietary Cu increased. The decreased rate of turnover of the slow turnover pool when intake was low aided in conserving body Cu stores.
45.3 Conclusion	Regulation of absorption and of endogenous excretion in response to dietary Cu intake helps to protect against deficiency and toxicity.
45.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
45.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of Cu 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 Cu 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: 5.0/08	**A6.2(08), Homeostasis 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	[redacted]
Reference	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	[redacted]

COMMENTS FROM ...

Date	*Give date of comments submitted*

Table A6.2(08)-1. Experimental Design

	Metabolic period		
	1	2	3
Duration (days)	24	42	24
Dietary copper (mg/day)	0.66	0.38	2.49
Study days in which ^{65}Cu was included in the diet of subjects 1-6	13-14[1]	31-32, 55-56[2]	79-80[1]
Study days in which ^{65}Cu was infused into subjects 7-12	13	55	79

[1]Copper was replaced with ^{65}Cu in the diet.
[2]0.2 mg ^{65}Cu/day was added to the diet.

Table A6.2(08)-2. Copper absorption, excretion and retention

Metabolic period	Dietary copper	Copper absorption (n = 4)		^{65}Cu excretion[2] (n = 6)	True absorption[3] (n = 4)		Copper retention (n = 11)
	mg/day	%	mg/day	%	%	mg/day	mg/day
1	0.66	54	0.35	26[a]	73	0.48	-0.13[a]
2	0.38	67	0.26	12[b]	77	0.29	-0.015[b]
3	2.49	44	1.08	34[a]	66	1.64	511[c]
SE[5]	--	3.2	--	3.8	--	--	39

[1]Means within a column with different superscript letters are significantly different, P > 0.05.

[2]Percentage of dose of ^{65}Cu excreted in 12days after infusion.

[3]Estimated from average absorption of fed ^{65}Cu adjusted for average excretion of infused ^{65}Cu.

[4]Average over entire metabolic period.

[5]Standard error of least-squares mean.

Table A6.2(08)-3. Endogenous gastrointestinal (GI) losses of copper at three intakes of dietary copper

Metabolic period	Dietary copper	Faecal copper[1]	Endogenous GI losses		
			Slow pool[2]	Fast pool[3]	Total[4]
		mg/day			
1	0.66	0.65	0.34	0.13	0.47
2	0.38	0.33	0.21	0.036	0.24
3	2.49	2.17	0.78	0.55	1.33

Table A6.2(08)-3. Endogenous gastrointestinal (GI) losses of copper at three intakes of dietary copper

[1]Last 6 days of each metabolic period (n = 11).

[2]Slow turnover pool (endogenous faecal losses not attributed to excretion of dietary copper in the first 12 days after absorption).

[3]Fast turnover pool (losses of dietary copper consumed and absorbed in the previous 12 days).

[4]Total endogenous losses (faecal copper less unabsorbed dietary copper).

Figure A6.2(08)-1

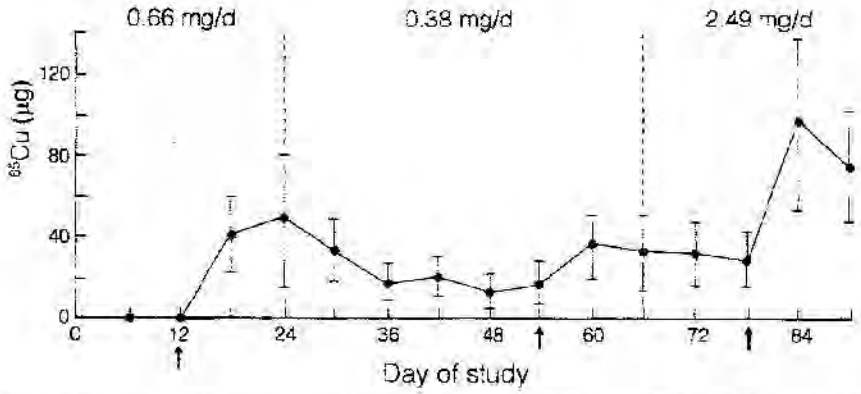

FIGURE 1. Excretion pattern of ^{65}Cu into the gastrointestinal tract after infusion of 0.34 mg of the isotope once in each dietary copper period ($\bar{x} \pm SD$, $n = 6$). Arrows indicate times of isotope administration. Vertical dotted lines represent changes in dietary copper intake, which was 0.66, 0.38, and 2.49 mg/d.

Figure A6.2(08)-2

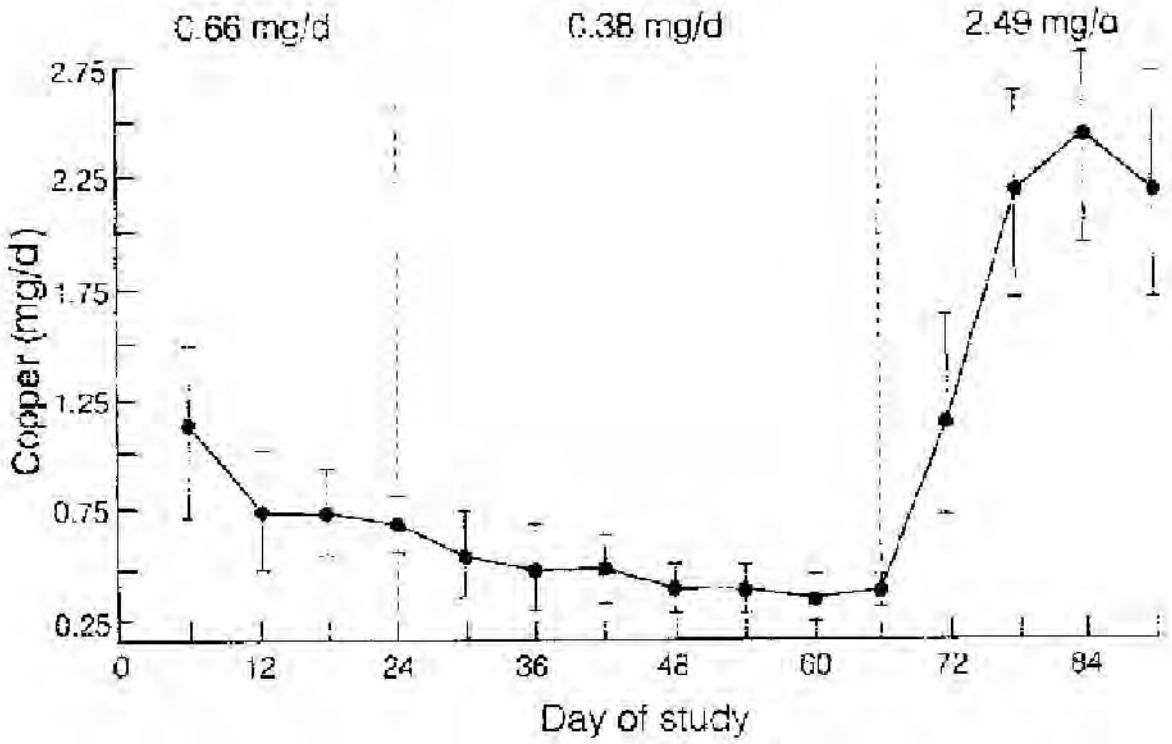

Figure A6.2(08)-3

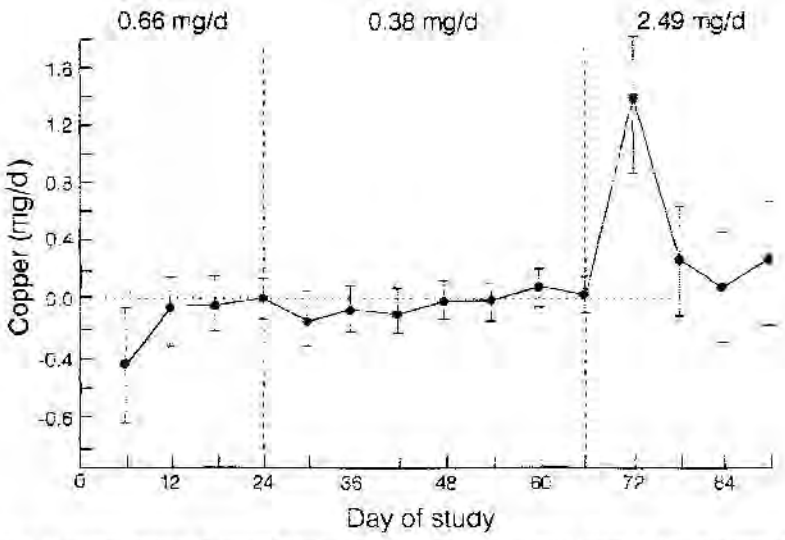

FIGURE 3. Copper balance (mg/d). Pattern of copper balance throughout the study at three levels of dietary copper shown at the top of the graph ($\bar{x} \pm SD$, $n = 11$). Vertical dotted lines represent changes in dietary copper.

			Official use only
		46 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).* Linder M.C., Weiss K.C. and Hai, V.M. (1988). Structure and function of transcuprein in transport of copper by mammalian blood plasma. In: Hurley L.C., Keen C.L., Lonnerdal, B. and Rucker, R.B. (eds). Trace Elements in Man and Animals (TEMA-6). New York: Plenum, 141–144 (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	
		47 GUIDELINES AND QUALITY ASSURANCE	
47.1	**Guideline study**	No. This was a non-regulatory study carried out to investigate the structure and function of the copper transport proteins transcuprein and albumin. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
47.2	**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)*	
47.3	**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")*	
		48 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.*	
48.1	**Test material**	Cu (II) as either the chloride or as Cu-nitrilotriacetate complex.	
48.1.1	Lot/Batch number	Not available	
48.1.2	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: 5.0/09	**A6.2(09), Distribution of copper**

48.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Not available	
48.1.2.2	Purity	*Give purity in % of active substance* [redacted]	X
48.1.2.3	Stability	*Describe stability of test material* Not available	
48.1.2.4	Radiolabelling	*give structural location of radio labelling, give reason if not labelled* ^{67}Cu	
48.2	**Test Animals**	Non-entry field	
48.2.1	Species	Plasma samples used in this study were obtained from rats or humans, as appropriate.	
48.2.2	Strain	Not available	
48.2.3	Source	Not available	
48.2.4	Sex	Not available	
48.2.5	Controls	No	
48.3	**Investigations**	Non-entry field	
48.3.1	Purification and structure of transcuprein.	Partial purification of transcuprein was carried out with a variety of procedures, including ammonium sulphate fractionation, where transcuprein precipitates between 35 – 50% saturation; gel permeation chromatography on Sephadex G150, where it elutes in the void volume and on Ultrogel AcA 34, where it elutes with an apparent molecular weight of 270 kDa; DEAE-cellulose chromatography, in phosphate, pH 7.0, where it elutes at about 0.15M NaCl in a 0 – 0.5M gradient; and pseudoaffinity chromatography (Affigel Blue).	
48.3.2	Properties and copper binding.	The specific binding of copper to partially purified transcuprein was measured in a nitrocellulose filter assay, after incubation of the protein with various concentrations of ^{67}Cu, in the presence and absence of a 100-fold molar excess of non-radioactive Cu-NTA at pH 7.0 in 20 mM phosphate-buffered saline. The rate of Cu release from transcuprein was also studied in dialysis and compared with that of albumin. Samples (2.0 ml) of radioactively labelled protein obtained from Sephadex G150 chromatography of ^{67}Cu-treated rat plasma (containing 1.5 – 2 million cpm) were placed in 500 ml buffer and dialysed at 4°C. Aliquots (5.0 ml) were withdrawn periodically and measured for release of radioactive Cu. At the end of dialysis, the ^{67}Cu remaining in the dialysis tubing was also measured.	

48.3.3 Detection of transcuprein by in-vivo labelling and gel chromatography

The purpose of this study was to determine how to use in-vitro labelling to quantify the amounts of transcuprein present in samples, based on a known albumin content. This was done by:

1. Ascertaining what amounts of Cu (^{67}Cu or ^{64}Cu) could be added to samples in-vitro that would allow detection of transcuprein in the presence of various amounts of albumin.

2. Developing a method of using the proportion of radioactivity (relative to that on albumin) to assess transcuprein content. Cu (II) was added as either the chloride or the NTA complex to 1.0 ml samples of rat or human whole plasma or serum, or to 1.0 ml samples of partially purified rat transcuprein and albumin.

48.3.4 Transcuprein in portal blood.

The purpose of this investigation was to demonstrate that transcuprein is present in portal blood in similar or greater amounts to that in plasma taken from the blood of the vena cava, after passing through the liver. **Figure A6.2(09)-2** shows the radioactive labelling of transcuprein, albumin and low molecular weight components in portal plasma, upon administration of 62 ng Cu (as ^{67}Cu-NTA) to the lumen of a 7 cm, tied-off segment of the upper small intestine in an anaesthetised rat.

49 RESULTS AND DISCUSSION

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

49.1 Purification and structure of transcuprein.

Tracking transcuprein with ^{67}Cu and by its characteristic elution in Sephadex or Ultrogel, resulted in a preparation containing a major component with Rf 0.41 in disc PAGE (using a 5% separating gel and pH 8.8 Tris-glycine buffer). Three minor protein components were also present, with Rf values of 0.11, 0.24 and 0.60, respectively. The major component had subunits of Mr 80,000 after dissociation and electrophoresis in SDS.

49.2 Properties and copper binding.

Preliminary binding studies indicated that binding of Cu to transcuprein was saturable and half maximal in the range of 10^{-9} M. It was also found that albumin behaved similarly.

The results of dialysis indicated that, at pH7-8, Cu was released at a very slow, linear rate. The rate was, however, raised at higher and lower pH values.

These studies confirm that the affinity of transcuprein for Cu is similar to that of albumin.

49.3 Detection of transcuprein by in-vivo labelling and gel chromatography

It was found that the amount of Cu added to whole plasma or serum determined the relative proportions that bound to transcuprein and albumin (**Table A6.2(09)-1**). At the lowest concentrations (in the range 1 – 2 ng/ml), almost exactly one third bound to transcuprein and two thirds to albumin. Very little bound to other components that could be separated in gel chromatography. At concentrations almost 1000 times greater than this (2 or more µg/ml), radioactivity on transcuprein was proportionately very low. There was also prominent labelling of low molecular weight components.

When samples of partially purified transcuprein and albumin were mixed in various proportions, almost all of the radioactivity became associated with transcuprein at low albumin levels. The reverse was the case in the presence of increasing albumin levels (**Figure A6.2(09)-1**). It was concluded that the amount of radioactivity associated with the transcuprein peak in Sephadex G150 chromatography does reflect the amount of transcuprein present in the sample.

49.4	Transcuprein in portal blood.	More than one third of of the radioactivity in albumin + transcuprein from portal blood was found to be associated with the transcuprein. This is a greater proprtion than is found in samples of vena caval blood labelled in vitro. It was concluded that transcuprein is indeed present in the portal blood, and that it is present in similar or higher amounts than in blood leaving the liver.

50 APPLICANT'S SUMMARY AND CONCLUSION

50.1 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 structure and function of the copper transport proteins transcuprein and albumin. No guidelines are available to address this objective and the study was not carried out or reported in compliance with GLP.

Partial purification of transcuprein was achieved with a variety of procedures, including ammonium sulphate fractionation; gel permeation chromatography; DEAE-cellulose chromatography; and pseudoaffinity chromatography. The specific binding of copper to partially purified transcuprein was measured in a nitrocellulose filter assay, after incubation of the protein with various concentrations of ^{67}Cu in the presence and absence of a molar excess of non-radioactive Cu.

The rate of Cu release from transcuprein was studied in dialysis and compared with that of albumin. At the end of dialysis, the ^{67}Cu remaining in the dialysis tubing was also measured. The use of in-vitro labelling to quantify the amounts of transcuprein present in samples, based on a known albumin content, was done by 1) ascertaining what amounts of ^{67}Cu or ^{64}Cu could be added to samples in-vitro that would allow detection of transcuprein in the presence of various amounts of albumin, and 2) developing a method of using the proportion of radioactivity (relative to that on albumin) to assess transcuprein content.

The amounts of transcuprein, albumin and low molecular weight components in portal blood were compared with those in plasma taken from the blood of the vena cava, after passing through the liver.

50.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Tracking transcuprein with ^{67}Cu and by its characteristic elution in Sephadex or Ultrogel, resulted in a preparation containing a major component with Rf 0.41 in disc PAGE. This component had subunits of Mr 80,000 after dissociation and electrophoresis in SDS.

Preliminary binding studies indicated that binding of Cu to transcuprein was saturable and half maximal in the range of 10^{-9} M. It was found that albumin behaved similarly. The results of dialysis indicated that, at pH7-8, Cu was released at a very slow, linear rate. The rate was raised at higher and lower pH values. These studies confirm that the affinity of transcuprein for Cu is similar to that of albumin.

The amount of Cu added to whole plasma or serum determined the relative proportions that bound to transcuprein and albumin. At concentrations in the range 1 – 2 ng/ml, almost exactly **1/3** bound to transcuprein and **2/3** to albumin. At concentrations ≥2 µg/ml, radioactivity on transcuprein was proportionately very low. When samples of partially purified transcuprein and albumin were mixed in various proportions, almost all of the radioactivity became associated

	with transcuprein at low albumin levels. The reverse was true in the presence of increasing albumin levels. It was concluded that the amount of radioactivity associated with the transcuprein peak in Sephadex G150 chromatography does reflect the amount of transcuprein present in the sample. More than **1/3** of the radioactivity in albumin plus transcuprein from portal blood was found to be associated with transcuprein. This is a greater proprtion than in samples of vena caval blood labelled *in vitro*. It was concluded that transcuprein is present in portal blood at similar or higher amounts than in blood leaving the liver.
50.3 Conclusion	Absorbed copper initially binds reversibly to transcuprein and albumin and is transported via portal blood to the liver. Transcuprein and albumin are also present in vena caval blood.
50.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
50.3.2 Deficiencies	Yes. This study was not conducted and/or reported in compliance with GLP. When compared with generally accepted principles to be applied to toxicokinetics studies, as set out in OECD guideline 417, it is also apparent that methodological details were poorly reported in places, including: • Information on the animals from which samples of plasma were obtained for use in the study (eg. Numbers of animals used and the conditions in which they were housed); • Information on the laboratory techniques applied to isolate and detect transcuprein and albumin and to investigate their various properties. • Information on the specification of the test substance is deficient. These deficiencies do not, however, necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes and that the results obtained are consistent with work published by other researchers. Furthermore, this research (including its methodology) was published in a peer-reviewed publication, 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. *(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: 5.0/09	**A6.2(09), Distribution of copper**

Evaluation by Competent Authorities
[redacted]

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

Table A6.2(09)-1

Distribution of ^{67} Cu between transcuprein and albumin, upon addition to rat plasma, in vitro.

Copper added (ng) [1]	Percent Radioactivity on	
	Transcuprein	Albumin
1	35	65
20	31	69
200	28	72
2000	16	84
2700	2	98

[1] 0.1 ml 0.9%NaC1 containing various amounts of Cu (as ^{67} Cu-NTA; 1:1 molar ratio) added to 1.0 ml rat plasma 30-90 min before Sephadex G150 chromatography.

Figure A6.2(09)-1

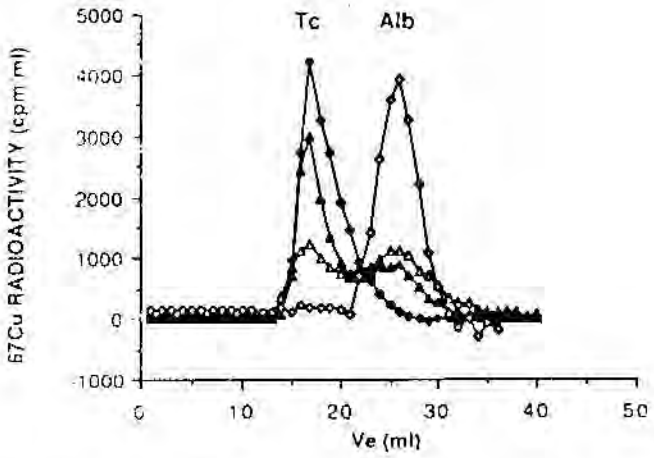

Fig. 1. Distribution of added ^{67}Cu radioactivity after in vitro addition of 2-20 ng Cu to partially purified transcuprein or albumin alone, or to various mixtures of the two. Sample applied to Sephadex G150.

Figure A6.2(09)-2

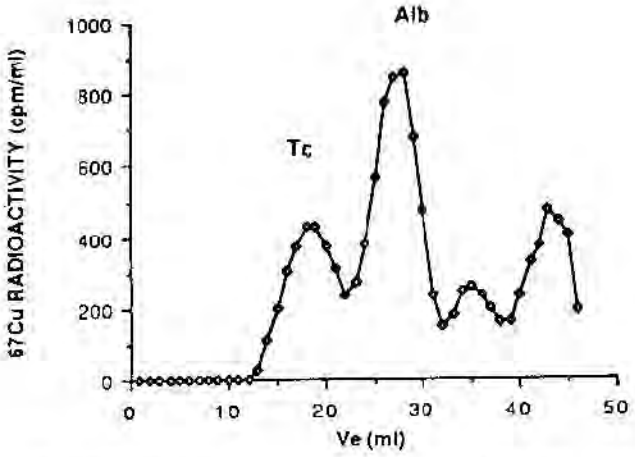

Fig. 2. Elution of copper binding components labeled with ^{67}Cu in portal blood plasma, 30 min after administration of radiocopper to a tied-off intestinal segment of an anesthetized rat. Plasma was applied to Sephadex G150 chromatography. The first peak elutes in the position of transcuprein, the second albumin.

			Official use only
		51 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).* Weiss K.C. & Linder M.C. (1985). Copper transport in rats involving a new plasma protein. Am. J. Physiol. **249**: E77-88 (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	
		52 GUIDELINES AND QUALITY ASSURANCE	
52.1	**Guideline study**	No. This was a non-regulatory study carried out to investigate the mechanism of copper transport in blood plasma following absorption from the diet. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
52.2	**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)*	
52.3	**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")*	
		53 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.*	
53.1	**Test material**	$CuCl_2$	
53.1.1	Lot/Batch number	Not available	
53.1.2	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: 5.0/10	**A6.2(10), Distribution of copper**

53.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Samples of ^{67}Cu were shipped in 1 – 5 M HCl and diluted in H_2O (for intragastric intubations of rats) or neutralised with NaOH in 0.9% NaCl just before injection.
53.1.2.2 Purity	*Give purity in % of active substance* [redacted]
53.1.2.3 Stability	*Describe stability of test material* See section 3.1.2.2
53.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* ^{67}Cu (see 3.1.2.2)
53.2 Test Animals	Non-entry field
53.2.1 Species	Rat
53.2.2 Strain	Fischer or Sprague-Dawley
53.2.3 Source	Simonsosn Labs, Gilroy, CA (Fischer) and Mission Labs, Rosemead, CA (Sprague-Dawley). (Human serum was primarily obtained frozen from Anaheim Memorial Hospital (Anaheim, CA). In some cases, fresh serum was obtained from laboratory volunteers).
53.2.4 Sex	Female
53.2.5 Controls	No
53.3 Investigations and procedures	Non-entry field
53.3.1 Animals, tissues and treatments	Adult, female Fischer or Sprague-Dawley rats were used in all animal studies. The copper status of the animals was maintained constant by keeping them on the same diet and using rats of similar age, sex, weight and strain. Where checked, tissue and blood analyses of copper gave constant values for different batches of rats. Animals were injected intraperitoneally, or intravenously by tail vein, with 0.1-0.4 ml $^{67}CuCl_2$ (10-300 µCi) in 0.9% NaCl, pH ~4, at various times before death. In some cases 0.2 to 0.3 m1 volumes of $^{67}CuCl_2$ in 0.01 N HCl were given by intragastric intubation. For the time course studies, only high specific activity radioisotope (~1,000 Ci/g) was employed (200-400 µCi/rat; 0.08-0.33 µg). In these studies, groups of six to nine rats were injected at the same and two to three killed at various times thereafter. Plasma was obtained from the vena cava, after anaesthetising with pentobarbital and injecting Na heparin solution. Rats were killed by exsanguination. Fresh plasma was used for most of the experimental

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	work. In some cases it was stored frozen at -20°C for several days. Human serum was obtained frozen from Anaheim Memorial Hospital (Anaheim, CA). In some cases, fresh serum was obtained from laboratory volunteers, on venusection.
53.3.2 Gel permeation chromatography	Samples of rat plasma or human serum were fractionated on 50- or 500 ml columns of Sephadex G150 or Ultogel AcA34 and equilibrated with 0.9% NaCl or 20 mM Na Phosphate, pH 7.0. For 50 ml columns, 1.0 ml samples were applied; 20 – 25 ml samples were used in the larger columns. For the Ultrogel, thyroglobulin, horse spleen ferritin, glutamate dehydrogenase, aldolase and albumin were used as molecular weight standards at concentrations of 2 mg/ml.
53.3.3 Chelex chromatography	Chelex 100 resin, as a slurry, was poured into microcolumns and prepared for copper chelation. After equilibration with pH 7.5 phosphate buffer, columns were tested for their ability to retain Cu^{2+}, either by applying 1 – 2 mg Cu^{2+} as $CuSO_4$ solution and analysing for copper by atomic absorption, or by applying $^{67}CuCl_2$ and checking for elution of radioactivity. In neither case was the copper released unless pH was lowered several units. Samples of plasma/serum or their extracts were applied under similar conditions; fractions were collected and analysed for radioactivity or for copper.
53.3.4 Assays of ceruloplasmin	Ceruloplasmin was assayed quantitatively as oxidase activity using p-phenylenediamine at pH 5.5. Qualitative detection was by double immunodiffusion in agarose plates or by immunoprecipitation in tubes using rabbit antiserum made against pure rat ceruloplasmin. For the former, 1.5% Agar Noble was dissolved by boiling in 0.9% NaCI and poured into calibrated plates. Merthiolate (0.01%) was added as a preservative. Wells held 10-20 µl antigen or antibody solution. Plates were incubated at room temperature for development of the precipitin bands. The effectiveness of antiserum was checked against pure protein or rat serum in the same plates. For immunoprecipitation, ^{67}Cu-labelled extracts were incubated in conical centrifuge tubes with varying amounts of antiserum in 0.9% NaCI (total vol 2.5 ml) overnight at 4°C, then centrifuged to collect precipitates, and the precipitates counted, then washed twice with 2.5 ml cold 0.9% NaCl. Supernatants and final pellets were also counted for ^{67}Cu radioactivity. Counts per minute in blanks containing no antiserum were subtracted from pellet counts per minute. The results were calculated as percent counts per minute precipitated.
53.3.5 Copper analysis	Copper analysis was carried out by furnace atomic absorption using a model 457 spectrometer. Except for tissue homogenates, analyses were carried out without prior wet ashing of samples. Samples of serum/ plasma not wet ashed were diluted 10-fold with 0.9% NaCl before analysis; column extracts were analyzed directly, without further dilution. For ashing, 0.10 to 0.50 ml samples were reduced in volume to 0.10 ml or less, by heat evaporation, and digested twice with 0.50 ml acid mixture, containing ultrapure nitric:sulphuric:perchloric acids (24:24:1), at 350-500°C. Residues were dissolved in 0.01 M H_2S0_4 before analysis.
53.3.6 Cell culture	Cultures of Ehrlich ascites' tumour cells and BALB C CL.3 fibroblasts were maintained in RPMI medium containing 6% bobby calf serum. For studies of copper uptake, cells were washed, scraped, counted, diluted, and transferred to the same medium without serum at a dilution of 10^6 cells per ml. Cells (1 ml aliquots) were incubated for 1 h in

serum-free medium (at 37°C, 5% $C0_2$), before copper protein solutions (30-150 µl in isotonic medium) were added. Uptake of copper was monitored by following uptake of radioactivity. After an additional hour of incubation, cells were chilled and separated from the medium (containing ^{67}Cu) by low-speed centrifugation, with two washings of 1.0 m1 cold 0.9% NaCl. All washes and the final pellet were counted. Uptake of Cu (in ng/10^6 cells) was calculated based on the specific activity (cpm/µg Cu) of the copper source. Controls were kept at 0°C during exposure to ^{67}Cu carriers, to permit binding but prevent internalization of the copper.

54 RESULTS AND DISCUSSION

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

54.1 Results

54.1.1 Evidence for a new copper transport protein in plasma (transcuprein).

When rats received intraperitoneal injections of tracer $^{67}CuCl_2$, or were given the isotope by intragastric intubation, fractionation of plasma components on columns of Sephadex G-150 showed two peaks of radioactivity within minutes of isotope administration (**Figure A6.2(10)-1A**). The second peak corresponded to albumin, eluting with the same volume as the albumin standard. The first peak eluted before ceruloplasmin, as shown by measuring p-phenylenediamine activity, and by the failure of a rabbit antiserum against ceruloplasmin to react with material in column fractions from the first peak (**Figure A6.2(10)-2**). Similarly, ^{67}Cu in the first peak was not precipitated by ceruloplasmin antibody.

Rechromatography of the first peak on ultragel revealed a single component (transcuprein) with an elution volume corresponding to a molecular weight of 270,000 Daltons (**Figure A6.2(10)-3**).

From these studies, it was apparent that newly administered copper first bound to ablumin and transcuprein in the plasma. With time, the proportion of total radioactivity in the two peaks diminished and a third peak, eluting in the position of ceruloplasmin, appeared between the two on columns of G-150 (**Figure A6.2(10)-1B**). By 24 h, only the central ceruloplasmin peak was radioactively labelled (**Figure A6.2(10)-1C**), and this could be precipitated with antibody against ceruloplasmin.

The data obtained from a large number of these experiments are summarised in **Table A6.2(10)-1**. These data are reported in terms of the proportion of total plasma radioactivity associated with each of the three plasma peaks, at isotope administration times ranging between 15 and 24 hours. Up to approximately one-third of the ^{67}Cu (representing new copper entering the body) was attached to transcuprein in the early phases of its distribution in the blood. The remainder was on albumin.

The proportion of label in transcuprein was less when ^{67}Cu preparations of lower specific activity were employed, suggesting that albumin was able to bind more Cu atoms because it is more abundant in plasma.

The distribution of stable copper among plasma and serum components fractionated on similar columns of Sephadex G-150 was considered. As shown in **Figure A6.2(10)-4**, analysis of fractions for copper by furnace atomic absorption spectrometry indicated that 10 – 15% of the total Cu of rat plasma was associated with transcuprien. Similar results obtained when serum vs. plasma of rats was analysed implied that the absence or

	presence of fibrinogen made no difference.	
54.1.2 Properties of transcuprien.	Transcuprien and albumin were readily labelled when traces of $^{67}CuCl_2$ were mixed with plasma or serum in vitro (demonstrated using Sephadex G-150 chromatography). In determinations with preparations of relatively higher or lower specific activity, proportions of ^{67}Cu in the transcuprein vs. albumin fractions corresponded to those obtained in vivo (15–17 vs. 83–95% respectively). When portions of either the ^{67}Cu-transcuprein or the –albumin peaks (obtained by G-150 chromatography) were infused into whole animals intravenously, plasma samples obtained 15 minutes later had the usual distribution of ^{67}Cu radioactivity found when $^{67}CuCl_2$ was injected. This indicated a rapid transfer of copper between transcuprein and albumin. The same phenomenon was demonstarted in vitro, when ^{67}Culabelled transcuprein or ^{67}Cu-albumin (from columns of Sephadex G150) were added to samples of whole rat plasma just before column fractionation. Irrespective of the source, the $^{67Cu2+}$ was distributed to transcuprein and albumin in the same proportions (**Figure A6.2(10)-5**). The copper of transcuprein was at least as firmly bound as that of albumin. Partially purified samples of transcuprein and albumin labelled with radiotracer were applied to columns of Chelex 100, a resin with a high affinity for Cu^{+} at pH 7.5. No radioactivity was retained by the columns. Further evidence for the tenacity of copper binding to transcuprein was also obtained by assessing the retention of radioactivity by the protein on its dilution before column fractionation. Tenfold dilution resulted in no decrease in the percentage of applied counts per minute recovered on transcuprein, whereas the recovery on albumin decreased from 85 to 70%. To confirm that transcuprein itself could be a source of copper for cells and to make an initial comparison of its capacity to donate copper with that of ceruloplasmin and albumin, fractionated samples of all three components (from plasma of $^{67}CuCl_2$-treated rats) were incubated with two different cultured cell lines, in vitro, in serum-free medium. As shown in **Table A6.2(10)-2**, some transcuprein bound to the cells , and bound better per picograms Cu added than did albumin. Both cell lines absorbed measurable quantities of copper from all three components over 1 hour. Per picogram Cu added, more transcuprein copper was absorbed than copper from ceruloplasmin or albumin.	X
54.1.3 Time course of copper distribution among plasma components and tissues after its administration.	The distribution of ^{67}Cu to plasma components and solid tissues was investigated (as % dose administered) following intravenous and intraperitoneal administration of the radioisotope. After administration, tracer $^{67Cu2+}$ was initially in the blood (**Figure A6.2(10)-6**), but by 6 hours was transferred largely to the liver and kidney. Thereafter, radioactivity re-emerged rapidly in the plasma and also accumulated in the peripheral tissues. With time, levels of tracer decreased in all organs. During the initial period, radiolabel in the blood was associated with transcuprein and albumin (**Figure A6.2(10)-1A**). When the tracer re-emerged in the plasma, the label was on ceruloplasmin (**Figures A6.2(10)-1C** and **A6.2(10)-7**). The total radioactivity in ceruloplasmin decreased rapidly after 24 hours (**Figure A.6.2(10)-7**) as that in the liver also decreased (**Figure A6.2(10)-6**). From 24 hours to 12 days, Sephadex G-150 chromatography showed the diminishing ^{67}Cu in plasma to be associated almost entirely with ceruloplasmin (**Figure**	

A.6.2(10)-7).

The specific radioactivity of copper in plasma components and in tissues was calculated for the various time points examined, based on relative counts per minute per gram and analyses of stable copper in rat tissues by furnace atomic absorption. The amounts in transcuprein, ceruloplasmin, and albumin were calculated from analyses of plasma fractionated on Sephadex G-150. When multiplied by organ/tissue weight, liver, kidney and blood were identified as major sites of copper deposition. Despite its lower copper concentration, skeletal muscle contained the greatest total mass of copper.

The results of five separate experiments, spanning the period from 5 minutes to 10-12 days after tracer administration, were combined by calculating counts per minute per gram tissue relative to the value for livers of rats killed at 24 hours in the same experiment (cpm/g liver at 24 hours = 100). Relative cpm/g tissue were then converted to relative cpm/μg Cu, using mean values for tissue Cu. Log mean specific activities of ^{67}Cu in Cu pools were plotted as a function of time after tracer administration (**Figure A6.2(10)-8**).

Immediately after i.v. or i.p. injection, the specific activities of albumin and transcuprein were very high, and that of albumin was slightly higher than transcuprein. The specific activities of both plasma components decreased rapidly, as those of liver Cu and kidney Cu increased. The liver attained its peak specific activity between 6 and 24 hours, and the kidney a little earlier. The specific activity of ceruloplasmin was then seen to incease, as that of liver decreased.

As the specific activity of ceruloplasmin Cu decreased over the next week, that of heart and skeletal muscle reached broad peaks between the 1st and 5th day, before decreasing very gradually. The peak for brain occurred after day 3, and showed no decrease even by days 10-12.

The decrease in specific activities of ceruloplasmin, liver and kidney followed an exponential course with apparent half-lives of 2.4, 4.2 and 4.6 days, respectively. Turnover of transcuprein and albumin copper was much more rapid, with half-lives of minutes. This decrease did not follow first-order kinetics, as half-life increased progressively with time. The decrease in whole-body radioactivity decreased exponentially with a half-life of 4.4 days, in parallel with ^{67}Cu in liver and kidney.

54.2 Discussion

By the use of a copper radioisotope of high specific activity, it has been shown that copper follows a highly specific pathway from the time it enters the blood plasma of the rat. Administration of this tracer to rats in vivo, or to plasma or serum in vitro, shows that cupric ions bind directly to two components of blood plasma; transcuprein and albumin.

On entering the blood , ^{67}Cu achieved an immediate very high specific activity in both transcuprein and albumin. This decreased rapidly (apparent half-lives less than 15 minutes), reaching very low levels by 6 hours. Concomitantly, there was a rapid increase in the specific activity of liver and kidney copper pools. Some time after 6 hours, the specific activity in liver reached its peak. It then decreased exponentially (apparent half-life 4.2 days), whereas the specific activity of plasma ceruloplasmin increased. Ceruloplasmin specific activity reached its peak at ~24 hours, then decreased as well (apparent half-life 2.2 days).

As ceruloplasmin specific activity began to decrease, that of heart, skeletal muscle and brain continued to increase, with brain especially reaching a peak well after that of ceruloplasmin. As ceruloplasmin

appeared to be the only ^{67}Cu-labelled component in plasma at this time, it must have been the source of the labelled copper appearing in peripheral tissues from 24 hours onward. Moreover, as there was only minimal incorporation of ^{67}Cu into these tissues during the first 6 hours, the only period in which other components in the plasma were labelled in quantities approaching those in ceruloplasmin, it appears that ceruloplasmin was the principal source of copper taken up by peripheral tissues throughout. This contrasts with the findings for liver and kidney, which appeared to gain copper only during the first 6 hours, when transcuprein and albumin were the main or only labelled components. Thus, after entering the blood (as from the intestine after entering the diet), copper is transported to the liver and kidney on albumin and transcuprein. Conversely, later on, after its incorporation into ceruloplasmin through liver synthesis, newly absorbed copper is transported to peripheral tissues on ceruloplasmin. During these events, there was also a fairly rapid excretion of newly absorbed copper from the body, as indicated by the diminishing retention of ^{67}Cu over time. The half-life, corrected for decay of ^{67}Cu activity, was ~4.5 days.

Up to one third of the copper entering plasma for the first time was bound to transcuprein and the remainder to albumin. This copper was available to cells in the absence of albumin, as shown with cells in culture. Transcuprein appears to have a very high affininty for copper. Tenfold dilution did not dissociate a significant amount of ^{67}Cu from transcuprein; neither did filtration through Chelex 100 at physiologiocal pH. Furthermore, ^{67}Cu could be transferred to transcuprein by simple mixing.

The high affinity of transcuprein for copper, relative to that of albumin, was demonstrated by the fact that transcuprein sites were saturated at much lower concentrations of added tracer copper than were the albumin sites. Thus the specific activity of the $^{67}CuCl_2$ preparations used had a direct effect on the proportion of added Cu^{2+} that bound to transcuprein. With high specific activities a large proportion bound, whereas with sufficient nonradioactive copper present, binding of ^{67}Cu to transcuprein was not seen, This implied that the copper binding sites on transcuprein were readily saturable, and that in the presence of competing non-radioactive Cu^{2+} insufficient $^{67Cu2+}$ can bind for detection.

55 APPLICANT'S SUMMARY AND CONCLUSION

55.1 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 rats to investigate the mechanism of copper transport in blood plasma following absorption from the diet. No guidelines are available to address this objective, and the study was not carried out or reported in compliance with GLP.

Test animals were injected intraperitoneally, or intravenously by tail vein, with 0.1-0.4 ml $^{67}CuCl_2$ (10-300 µCi) in 0.9% NaCl, at various times before death. In some cases 0.2 to 0.3 m1 of $^{67}CuCl_2$ in 0.01 N HCl were given by intragastric intubation. For the time course studies, only high specific activity radioisotope (~1,000 Ci/g) was employed (200-400 µCi/rat; 0.08-0.33 µg). In these studies, groups of six to nine rats were injected at the same and two to three killed at various times thereafter. Rats were killed and plasma was obtained from the vena caval blood. Where appropriate, human plasma was obtained frozen

from hospital samples or fresh from laboratory volunteers.

Samples of rat plasma or human serum were fractionated on Sephadex G150 or Ultogel AcA34 columns and equilibrated with 0.9% NaCl or 20 mM Na Phosphate, pH 7.0. For the Ultrogel, thyroglobulin, horse spleen ferritin, glutamate dehydrogenase, aldolase and albumin were used as molecular weight standards at concentrations of 2 mg/ml.

Chelex 100 resin, as a slurry, was poured into microcolumns and prepared for copper chelation. After equilibration with pH 7.5 phosphate buffer, columns were tested for their ability to retain Cu^{2+}, either by applying 1 – 2 mg Cu^{2+} as $CuSO_4$ solution and analysing for copper by atomic absorption, or by applying $^{67}CuCl_2$ and checking for elution of radioactivity. In neither case was the copper released unless pH was lowered several units. Samples of plasma/serum or their extracts were applied under similar conditions; fractions were collected and analysed for radioactivity or for copper.

Ceruloplasmin was assayed quantitatively as oxidase activity using p-phenylenediamine at pH 5.5. Qualitative detection was by double immunodiffusion in agarose plates or by immunoprecipitation in tubes using rabbit antiserum made against pure rat ceruloplasmin.

Copper analysis was carried out by furnace atomic absorption using a model 457 spectrometer. Except for tissue homogenates, analyses were carried out without prior wet ashing of samples.

For studies of copper uptake, cultures of Ehrlich ascites' tumour cells and BALB C CL.3 fibroblasts were incubated for 1 h in serum-free medium (at 37°C, 5% $C0_2$), before copper protein solutions (30-150 µl in isotonic medium) were added. Uptake of copper was monitored by following uptake of radioactivity. After a further hour of incubation, cells were separated from the medium (containing ^{67}Cu) by low-speed centrifugation. All washes and the final pellet were counted. Uptake of Cu (in ng/10^6 cells) was calculated based on the specific activity (cpm/µg Cu) of the copper source.

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/10	**A6.2(10), Distribution of copper**

55.2 Results and discussion	*Summarize relevant results; discuss dose-response relationship* The time course of distribution of high specific activity $^{67}CuCl_2$ to tissues and plasma components was followed in adult female rats. Immediately after intubation or injection, tracer ^{67}Cu became associated with two components of the blood plasma separable on columns of Sephadex G-150; albumin and another component which was not ceruloplasmin. The latter component (transcuprein) had an apparent molecular weight of 270,000, and a high capacity for Cu^{2+}, as determined by processing through Chelex-100, dilution, and exchange with albumin copper , *in vitro* and *in vivo*. It was capable of donating copper to tumour cells in serum-free medium. Analysis of 'cold' plasma by furnace atomic absorption spectroscopy confirmed the presence of 10-15% of plasma copper in this peak. Plots of percent dose and ^{67}Cu specific activity against time showed that copper followed a very specific pathway after binding to albumin and transcuprein, entering mainly the liver, then reappearing in the plasma on ceruloplasmin, and then achieving peak distribution in peripheral tissues (muscles, brain, etc.). ^{67}Cu disappeared from liver and kidney with an apparent half-life of 4.5 days, the same exponential rate found for whole-body turnover. Apparent turnover of ceruloplasmin copper was more rapid. Even after 7 – 12 days, tracer copper in plasma was still found exclusively with ceruloplasmin.
55.3 Conclusion	These results indicate that copper follows a carefully prescribed path upon entering the blood and binding rapidly and strongly to the transport proteins transcuprein and albumin. Most of this bound copper is transported in the portal blood to the liver, although some goes directly to other tissues, especially the kidneys. Once in the liver, copper is incorporated into ceruloplasmin, which is subsequently released into the systemic circulation for delivery to other tissues.
55.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
55.3.2 Deficiencies	*(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)* Yes. This study was not conducted and/or reported in strict compliance with the principles of GLP. When compared with generally accepted principles to be applied to toxicokinetics studies, as set out in OECD guideline 417, it is also apparent that methodological details were poorly reported in places, including: • Information on the animals used in the study (eg. Numbers and characteristics of animals used and the conditions in which they were housed); 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: 5.0/10	**A6.2(10), Distribution of copper**

Evaluation by Competent Authorities
[redacted]

COMMENTS FROM ...

Date *Give date of comments submitted*

Table A6.2(10)-1

Distribution of ^{67} Cu among plasma components of the rat at various times after intraperitoneal injection of radioisotope.

	Transcuprein	Ceruloplasmin	Albumin	Low Molecular Wt
Elution vol, ml	17±1 (23)	22±1 (16)	28±2 (23)	35-50
^{67} Cu distribution, % total cpm applied				
15 min				
High SA	26±5 (5)	7±1(4)	69±5 (5)	
Low SA	7±5 (4)		92±5 (4)	1±0 (3)
2 h				
High SA	16±8 (5)	46±9 (5)	39±6 (5)	
Low SA	1,7 (2)	42,1 (2)	56,80 (2)	1,3 (2)
24 h				
High SA	1±0 (3)	93±3 (3)	6±3 (3)	

Values are means ± SD; no. of observations is in parentheses. Percentage of total radioactivity recovered in 4 copper binding components by procedures described in Fig 1. Results for rats injected with ^{67} Cu of high (>750 Ci/g) vs. low (<750 Ci/g) specific activity (SA) are reported separately.

Table A6.2(10)-2

Cell uptake and binding of 67 Cu-labelled plasma components in culture.

Parameters	Transcuprein	Ceruloplasmin	Albumin
Ehrlich ascites tumor cells			
Binding			
% of dose	1.4	0.45	1.0
pg/10^6 cells	25	74	94
Net uptake			
% of dose	3.1	0.79	1.5
pg/10^6 cells	31	52	47
Normal fibroblasts, BALB/C			
Binding			
% of dose	0.81	0.85	0.74
pg/10^6 cells	14	55	65
Net uptake			
% of dose	2.8	0.52	1.03
pg/10^6 cells	36	27	38

Samples (50**p**l) of ^{67}Cu labelled transcuprein, ceruloplasmin, or albumin (Fig.1, A and C) were incubated with 10^6 cells in serum-free medium for 1 h at 37 or 0° C. Samples contained 1.8, 16.1, and 9.4 ng of copper, in the form of the 3 components listed, respectively. Uptake (at 37°C) and binding (at 0°C) were monitored by measuring radioactivity in the washed cells and washes. Data are averages for duplicate samples. Net uptake (pg Cu) was calculated as total uptake (37°C data) minus binding (0°C data).

Table A6.2(10)-3

56 COPPER CONTENT OF RAT TISSUE AND PLASMA COPPER COMPONENTS

	Copper Content	
	Concentration jtg.g or ml	Total/160g rat, jtg
Plasma		
Total	1.20 ± 0.09 (5)	19*
Ceruloplasmin	0.72	11.5
Transcuprein	0.18	2.9
Albumin	0.18	2.9
Liver	4.0 ± 0.4 (5)	22
Kidney	7.5 ± 0.6 (5)	8.0
Heart	3.1 ± 0.2 (5)	1.6
Spleen	2.1 ± 0.1 (3)	1.9
Skeletal muscle	1.1 ± 0.1 (3)	53†
Brain	2.5 ± 0.3 (3)	3.7

Values are means ± SD for adult female Fischer rats weighing 160 ± 7g. Number of observations is in parentheses. *Assuming blood is 10% body wt, 50% hematocrit. † Assuming muscle is 30% body wt.

Figure A6.2(10)-1

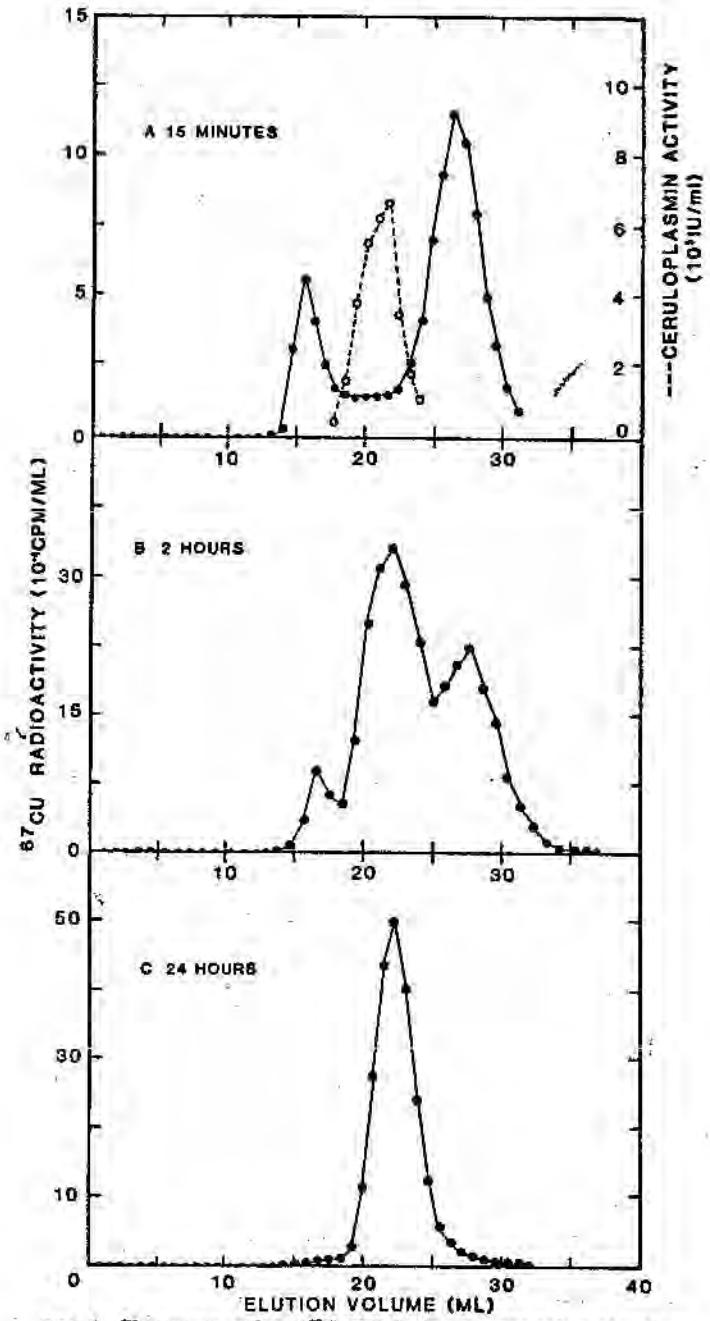


FIG. 1. Chromatography of ^{67}Cu-labeled rat plasma taken at various times after radioisotope administration. Pairs of rats were injected with 250–400 μCi of $^{67}CuCl_2$ in 0.9% NaCl and killed after 15 min (*A*) and 2 (*B*) and 24 h (*C*). Pooled samples (1.0 ml) of heparinized plasma from 2 rats (killed at same time intervals) were fractionated on 50-ml columns of Sephadex G-150. Radioactivity of 1.0-ml fractions is plotted against elution vol (ml) (*solid line*). *Dashed line* in *A* shows the elution of ceruloplasmin oxidase activity, measured with *p*-phenylenediamine (MATERIALS AND METHODS). Typical results for 1 time course study are shown.

Figure A6.2(10)-2

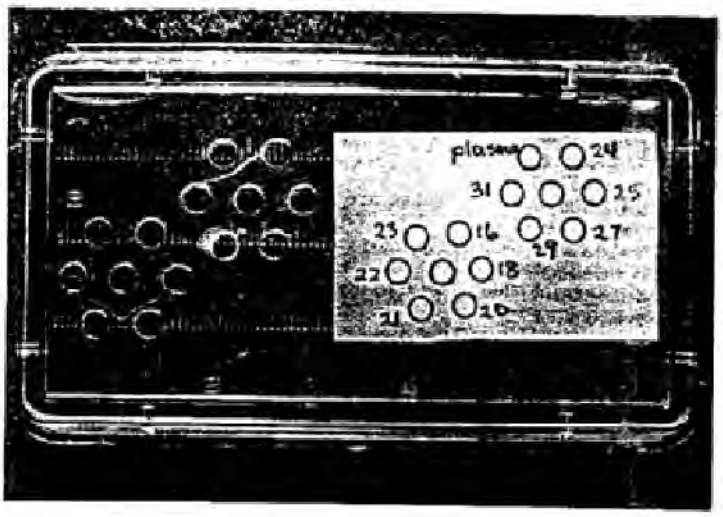


FIG. 2. Immunological identification of ceruloplasmin in column fractions by double immunodiffusion. Antibody made in rabbits against pure rat ceruloplasmin (20 μl) was placed in central well of each hexagonal rosette. Aliquots (20 μl) of fractions from Sephadex G-150 chromatography of rat plasma (Fig. 1A) were placed in peripheral wells; normal rat plasma (20 μl) served as a control for antibody-antigen interaction. Numbers refer to fractions (elution vol) from similar columns to those in Fig. 1. Typical result.

Figure A6.2(10)-3

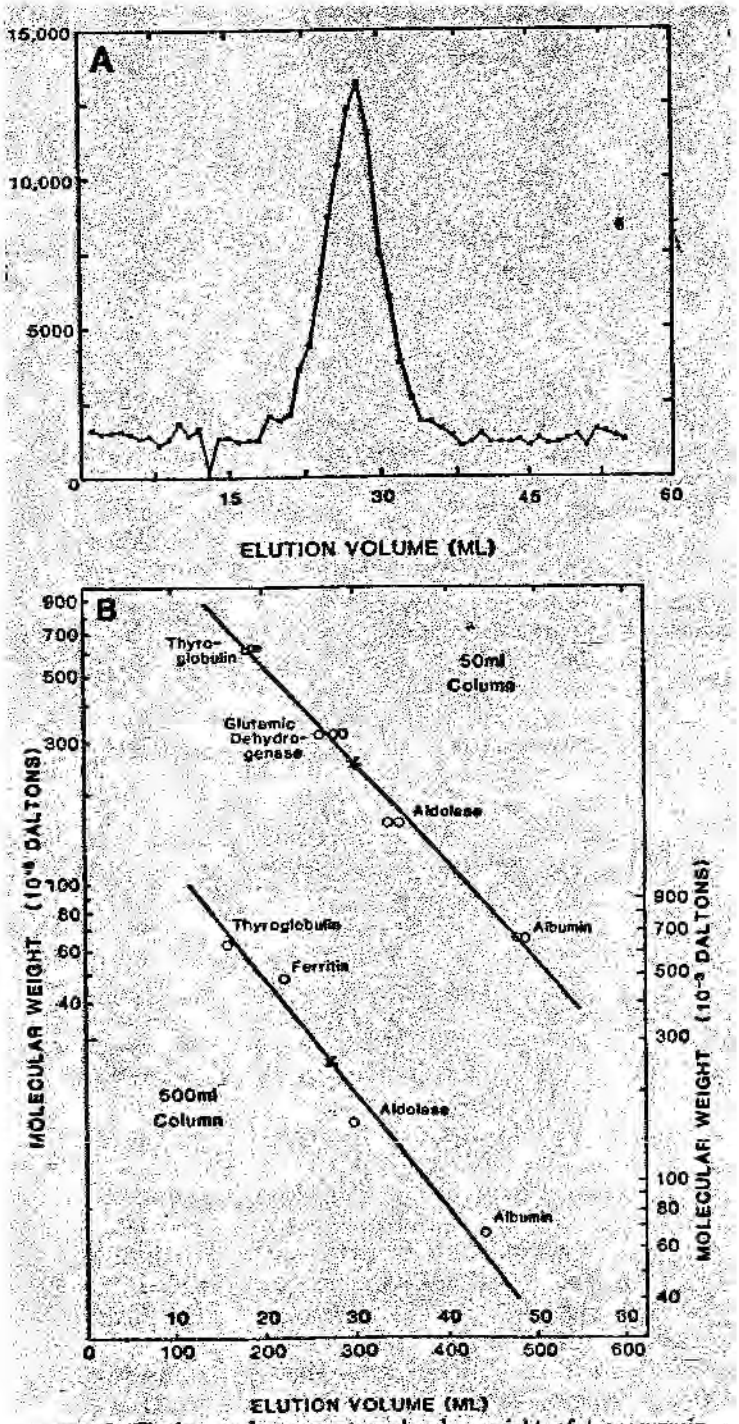


FIG. 3. Elution and apparent molecular weight of transcuprein, using Ultrogel AcA34. *A*: samples (2.5 ml) of ^{67}Cu-labeled transcuprein, obtained from chromatography of plasma on Sephadex G-150 (1st radioactive peak, Fig. 1A), were fractionated on 50-ml columns of Ultrogel AcA34. Elution of ^{67}Cu radioactivity (cpm/ml) is plotted against elution vol (ml). *B*: combined results of several chromatographic studies in which ^{67}Cu-labeled transcuprein samples were applied to and eluted from 50- and 500-ml columns calibrated with various standard proteins of known molecular weight. *Solid lines* indicate standard curves obtained with protein standards used (*C*). Stars (*) mark positions in which transcuprein samples eluted.

Figure A6.2(10)-4

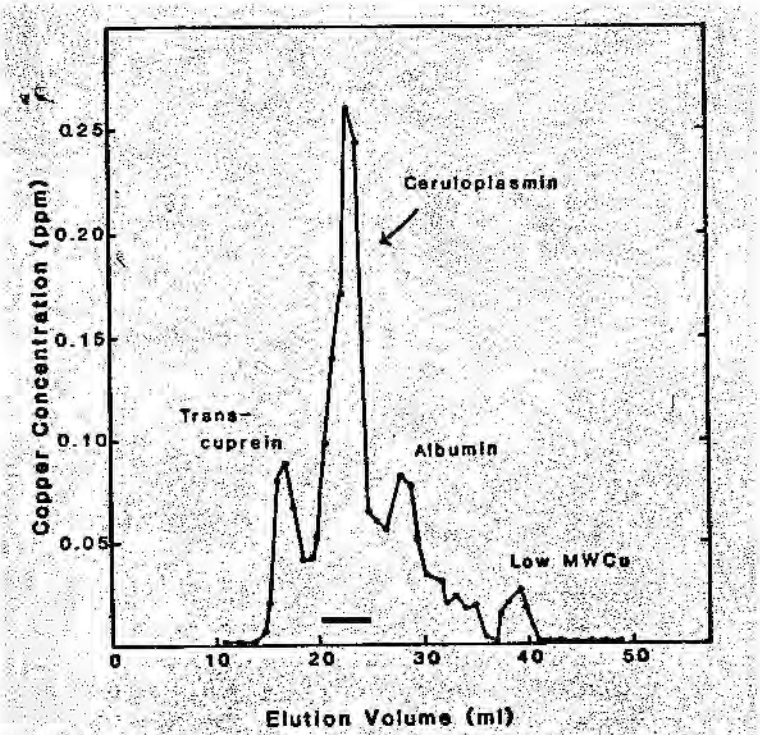


FIG. 4. Separation of copper components of plasma on Sephadex G-150. Samples (1.0 ml) of heparinized plasma from adult female rats were fractionated on 50-ml columns of Sephadex G-150 equilibrated with 20 mM phosphate. Fractions (1.0 ml) were assayed directly for copper content, by furnace atomic absorption spectroscopy. A typical profile for pooled rat plasma is shown. Amounts of copper in transcuprein, ceruloplasmin, and albumin were calculated from copper contents of indicated fractions. Position of ceruloplasmin (*p*-phenylenediamine oxidase activity) is indicated by *bar*.

Figure A6.2(10)-5

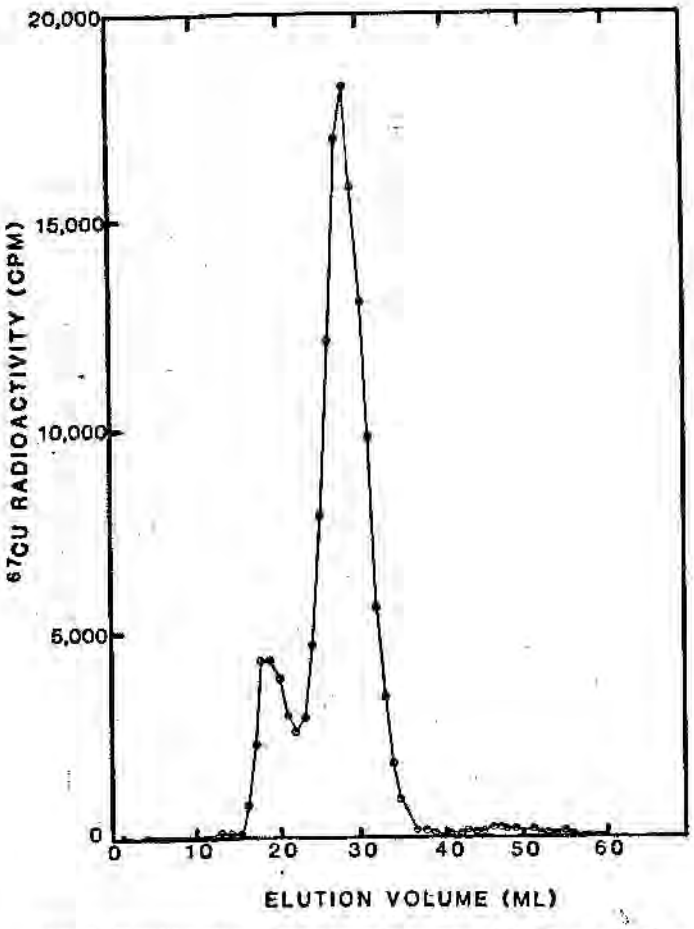


FIG. 5. Equilibration of transcuprein copper with albumin in vitro and vice versa. ^{67}Cu-labeled transcuprein (0.5 ml), obtained by fractionating plasma from rats injected with tracer $^{67}CuCl_2$ 15 min before death on columns of Sephadex G-150 (Fig. 1*A*), was mixed with an equal volume of cold plasma and applied to a similar 50-ml Sephadex column. Elution of ^{67}Cu radioactivity (cpm/fraction or per ml) is plotted against elution vol (ml). A typical result is shown. Similar profiles were obtained by mixing ^{67}Cu-albumin fractions with cold plasma and by adding $^{67}CuCl_2$ to cold plasma.

Figure A6.2(10)-6

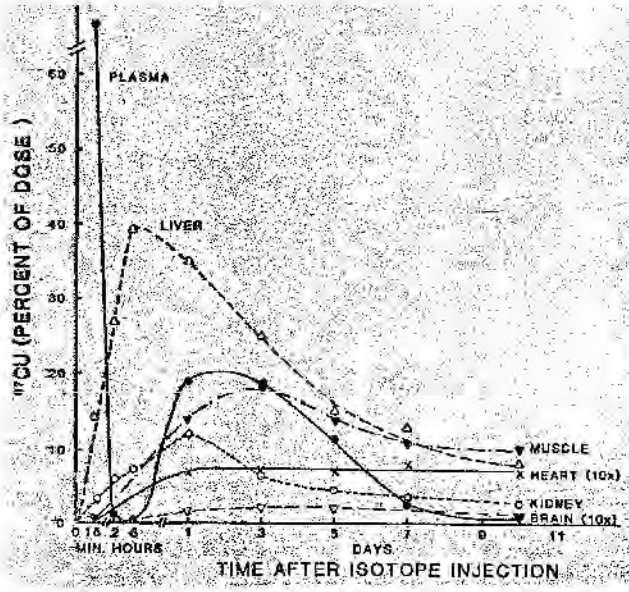


FIG. 6. Distribution of ^{67}Cu in rat tissues and plasma at various times after tracer administration. Radioactivity in plasma (●), liver (△), kidney (○), heart (×), muscle (▼), and brain (▽) was determined by counting whole organs or representative tissue samples from rats killed at various times after tracer administration. Values shown are percent dose, based on data for cpm/g (or per ml) and organ weights of animals. For skeletal muscle, a value of 30% body weight is assumed; for plasma, 5% of body wt. Results are for 4 studies involving following numbers of rats at each sequential time point: 5, 2, 2, 7, 7, 7, 3, 4. Eleven-day value is mean for 2 pairs of rats killed on *days 10* and *12*. *Points* are mean values. Coefficients of variation (SD/mean × 100) were less than 20% (mostly 10%), with exception of muscle at 15 min, kidney and plasma at 24 h, and liver at *day 5* (all under 25%).

Figure A6.2(10)-7

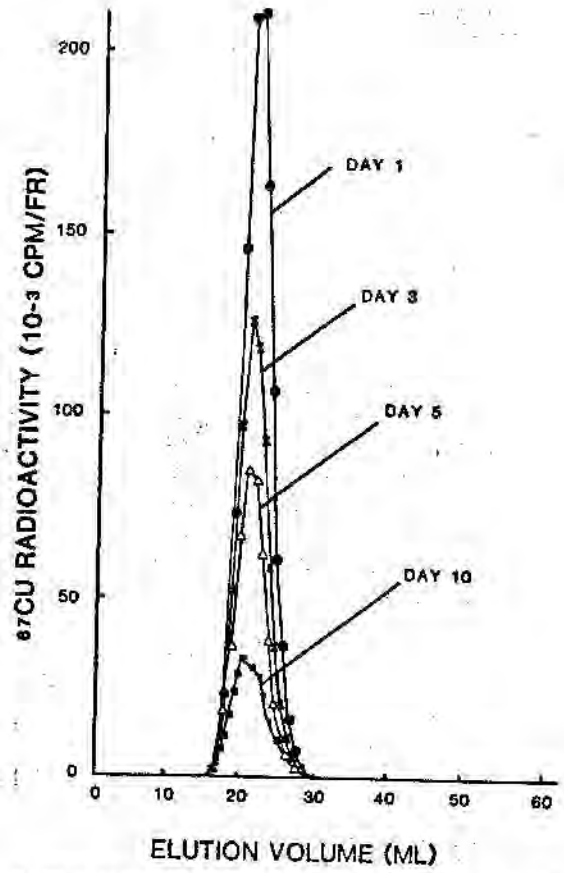


FIG. 7. Time course of ^{67}Cu-labeling of plasma components after 24 h. Samples of fresh plasma, taken from rats 1 to 10 days after administering $^{67}CuCl_2$ tracer, were fractionated on columns of Sephadex G-150 as described for Fig. 1. Rats killed at 1 (●), 3 (×), 5 (△), and 10 (□) days.

Figure A6.2(10)-8

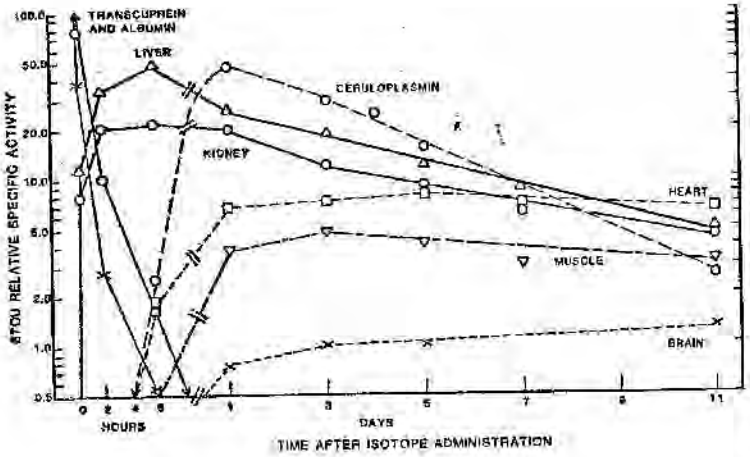


FIG. 8. Specific activity of co components and tissues at various ti after ^{67}Cu tracer administration. D for ^{67}Cu radioactivity (cpm/g or cp ml), relative to the value for liver at 1 h, were recalculated as relative specifi activities of copper in these various com partments (cpm/μg Cu) based on tissue copper concentrations given in Table 3. Log specific activities of copper in albumin (o—o), transcuprein (×—×), liver (Δ—Δ), kidney (o—o), ceruloplasmin (o—o), heart (□—□), muscle (▽—▽), and brain (×—×) are plotted against time after tracer administration, to indicate pathway copper takes on entry into body of the rat. Results are from 5 studies of groups of 2–3 rats, killed 15 min, 24 h, and 3, 5, 7, 10, and 12 days after tracer administration. *Points* shown are mean values for all rats killed at same time point. Data for *days 10* and *12* were combined and plotted as *day 11*. Total number of rats at each time point in sequence was as follows: 9, 4, 4, 9, 4, 7, 3, 4. Coefficients of variation (SD/mean × 100) were under 20% (usually ~10%) except for 15-min values (up to 36%).

			Official use only
		57 REFERENCE	
57.1	**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).* Lee S.H., Lancey R., Montaser A., Madani N., Linder M.C. (1993). Ceruloplasmin and copper transport during the latter part of gestation in the rat. Proc Soc Exp Biol Med **203**: 428-39 (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	
		58 GUIDELINES AND QUALITY ASSURANCE	
58.1	**Guideline study**	No. This was a non-regulatory study carried out to determine whether ceruloplasmin or ionic copper (binding to albumin + transcuprein) or both are the best maternal blood sources for transfer of copper to the foetus, by what mechanism that transfer occurs, and the initial fate of the copper in the foetus after transfer. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
58.2	**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)*	
58.3	**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")*	
		59 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.	
59.1	**Test material**	$^{67}CuCl_2$	
59.1.1	Lot/Batch number	Not available	
59.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

59.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* $^{67}Cu\ Cl_2$ was obtained in millicurie quantities with specific activities in the range of 4000 µCi/µg, upon receipt. Smaller quantities of noncarrier-added product, made from ^{67}Zn, were also utilised.
59.1.2.2 Purity	*Give purity in % of active substance* [redacted]
59.1.2.3 Stability	*Describe stability of test material* See section 3.1.2.1.
59.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* $^{67}Cu\ Cl_2$ (see section 3.1.2.1).
59.2 Test Animals	*Non*-entry *field*
59.2.1 Species	Rat
59.2.2 Strain	Sprague-Dawley
59.2.3 Source	Simonson Laboratories (Gilroy, CA) and Lab Pets (Riverside, CA).
59.2.4 Sex	Female
59.2.5 Treatment of animals	Pregnant rats were received mid-way through pregnancy and infused intravenously into the tail vein with ^{67}Cu-labelled samples under light pentobarbital anaesthesia. Rats were sacrificed by exsanguination under heavy anaesthesia, with heparin treatment. In one study, rats were treated with cycloheximide 30 minutes before and 1.75 hours after intravenous injection of ^{67}Cu(II)-treated serum to inhibit formation of ^{67}Cu-ceruloplasmin by maternal tissues. Whole blood was collected from the vena cave in the thoracic cavity and centrifuged to obtain plasma. Other organs removed were the liver, kidney, spleen, heart, brain, placentae and uterus. Half of the foetuses were taken for determination of their radioactivity. The other foetuses were used as sources of foetal blood and liver. In most cases, amniotic fluid was also collected by syringe before removal of foetuses. To obtain samples for determination of ceruloplasmin mRNA or membrane receptors, tissues were immediately chilled in ice-cole phosphatebuffered saline solution before homogenisation in appropriate buffers.
59.3 Procedures	Non-entry field
59.3.1 Preparation of samples	For preparation of ^{67}Cu-labelled ceruloplasmin, a donor rat was injected with 3-5 mCi of ^{67}Cu in the form of neutralised nitrilotriacetate (NTA) complex (Cu:NTA; 1 mole: 1 mole). Twelve to 19 hours later, the donor rat was sacrificed and 1.0 ml portions of plasma were fractionated on 50-ml columns of Sephadex G-150. A single radioactive peak was obtained that gave a single radioactive band in polyacrylamide gel electrophoresis characteristic of ceruloplasmin. The three most radioactive fractions were pooled for tail vein injection into animals. For preparation of ^{67}Cu-labelled albumin + transcuprein, 2 – 3 ng of Cu (labelled with ^{67}Cu(II)-NTA) was added to 1.0 ml portions of rat plasma 2 – 50 hours before injection. Binding of radioisotope to serum components fractionated on Sephadex G-150 was identical, irrespective of whether serum samples were first brought to pH 5.5 and back to 7.0 before injection.

	Radioactive samples were counted in a multisample gamma counter. Individual foetuses, foetal livers, placentae, and maternal organs were counted directly, as were samples of serum, column fractions, etc.
59.3.2 Chromatography	Sephadex G-150 chromatography was on 50 ml or 25 ml columns. Samples of 1.0 ml or 0.5 ml were applied to the larger and smaller columns, respectively. Fractions of 1.0 or 0.5 ml were collected for counting and other analyses.
59.3.3 Ceruloplasmin assays	Ceruloplasmin oxidase activity was measured using p-phenylene diamine as a substrate. In some cases, ceruloplasmin was identified by immunoprecipitation with specific rabbit anti-rat ceruloplasmin polyclonal antibody. Ceruloplasmin samples were also identified by their characteristic migration (versus bromophenol blue) in disk gel electrophoresis on polyacrylamide. Gels were either stained with amido black or sliced and counted for ^{67}Cu.
59.3.4 Ceruloplasmin receptor assays	Receptor assays were carried out in the presence and absence of an excess of nonradioactive (300 µM) Cu (II) (1:1 molar NTA complex). This concentration provides maximum competition in the binding assay. For this, portions (1 – 3 mg) of membrane protein were incubated with 50 pmol of ^{67}Cu-labelled ceruloplasmin in a total volume of 900 µl for 1 hour at room temperature, before separation of bound and free ceruloplasmin by Airfuge. Total binding and binding in the presence of excess Cu-NTA were recorded. To demonstrate binding kinetics, larger (25 – 50 mg) portions of membrane from placenta were incubated with varying amounts of ^{67}Cu-ceruloplasmin in the absence and presence of a 100-fold excess of non-radioactive ceruloplasmin, or "cold" Cu-NTA.
59.3.5 Ceruloplasmin mRNA	Total ceruloplasmin mRNA was determined using extracts of total RNA obtained from portions of guanidine thiocyanate-treated tissue. The distribution of ceruloplasmin-mRNA to free and endoplasmic reticulum-bound polyribosomes was also examined. For this, postnuclear supernatants were fractionated on discontinuous sucrose gradients, and total RNA was extracted from the appropriate fractions. For mRNA determinations, portions of RNA were slotblotted and hybridised with [^{32}P]cDNA for rat ceruloplasmin. Densitometry of autoradiographs developed from the slot blots was performed with a Beckman model 24 spectrophotometer with a scanner. Control blots were made with [^{32}P]cDNA for ferritin and tubulin.
	60 RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are given below.*
60.1 Results	Transport of copper from mother to foetus was studied in Sprague-Dawley rats 1 – 4 days before the end of gestation. The characteristics of these rats are summarised in **Table A6.2(11)-1**.
60.1.1 Uptake of copper from ceruloplasmin versus albumin and transcuprein.	Radioactive copper in the form of *in vivo*-labelled ^{67}Cu-ceruloplasmin or ^{67}Cu (II)-treated plasma was injected i.v. into rats 1 and 4 hours before death. **Figure A6.2(11)-1** shows how the samples used for injection chromatographed on Sephadex G-150, and how radioactivity was distributed among components of maternal serum at the two time-points examined. In the case of the *in-vivo*-labelled ceruloplasmin, plasma samples were taken from donor rats 12 – 19 hours after an i.p. injection of a large dose of ^{67}Cu (II). Fractionation on a column of Sephadex G-

150 gave a single peak eluting in the position of ceruloplasmin oxidase activity (**Figure A6.2(11)-1A**), and a single band migrating in the position of ceruloplasmin in polyacrylamide gel electrophoresis. Radioactivity in maternal serum taken 1 or 4 hours after intravenous injection of peak fractions of this ^{67}Cu-ceruloplasmin also eluted as a single symmetrical peak in the position of ceruloplasmin (**Figure A6.2(11)-1B**) and could be precipitated with rabbit, anti-rat ceruloplasmin antiserum.

Fresh plasma treated *in vitro* with ng quantities of ^{67}Cu-NTA, to label albumin and transcuprein, gave a different chromatographic distribution of the radiolabel (**Figure A6.2(11)-1C**). About one third of the radioactivity was on a peak in the void volume representing transcuprein, and the rest was on a peak eluting in the position of albumin standard. One hour after i.v. injection of this solution (**Figure A6.2(11)-1D**), the ^{67}Cu in the maternal serum was still mainly with transcuprein and albumin, but more was with the transcuprein fraction. By 4 hours, however, most was with a peak eluting in between, namely with ceruloplasmin, representing that synthesised by the pregnant rat from the injected ^{67}Cu (II).

Distribution of radioactivity to maternal and foetal tissues from the two different injected sources was compared. The data on uptake as % of dose are summarised in **Table A6.2(11)-2** and **Table A6.2(11)-3**. Values are based on total radioactivity per organ, 1 and 4 hours after injection of ^{67}Cu-ceruloplasmin or ^{67}Cu-(transcuprein + albumin).

Four hours after administration of ^{67}Cu-ceruloplasmin, three quarters of the ^{67}Cu was still circulating in the maternal blood with the ceruloplasmin that had been injected (**Table A6.2(11)-2**). Four hours after injection of ^{67}Cu (II) bound to transcuprein and albumin (**Figure A6.2(11)-2**), more than 80% of the radioactivity had left the maternal blood (**Table A6.2(11)-2**). At 1 hour and increasingly with time, detectable amounts of radioactivity were transferred to the placenta and foetus from either copper source (**Table A6.2(11)-2** and **Table A6.2(11)-3**), but more was transferred in the case of injections of ^{67}Cu-ceruloplasmin. On the basis of % dose/organ or % dose/g, the placenta seemed to be as avid for copper as the liver when copper was given as ceruloplasmin, and less avid when it was given as ^{67}Cu (II) bound to albumin and transcuprein (**Table A6.2(11)-2**). There was a significant uptake of copper by the uterus from both copper sources (**Table A6.2(11)-3**), and accumulation of ^{67}Cu from ceruloplasmin was greater than from albumin + transcuprein for maternal heart, brain and spleen, and less for liver and kidney (**Table A6.2(11)-3**). Foetal blood and tissues also accumulated more radioactivity over time when ^{67}Cu was given as ceruloplasmin (**Table A6.2(11)-2** and **Table A6.2(11)-3**).

Copper uptake by tissues was calculated from the specific activity of the injected ^{67}Cu once it had entered and been mixed with the copper in the endogenous ceruloplasmin (or transcuprein + albumin) plasma pool. Calculations were carried out for some of the tissues using mean values for total ^{67}Cu per organ, estimates of the amounts of copper injected, and reasonable assumptions about plasma volumes and sizes of plasma copper pools (**Figure A6.2(11)-2**). Since the plasma pool of ceruloplasmin-copper into which ^{67}Cu was injected is roughly twice as large as that of albumin + transcuprein-copper, the data show that ceruloplasmin was seven or eight times more effective than transcuprein and albumin at delivering copper to the placenta and foetus. It was 2.5

	times as effective in the case of the uterus. The effect of inhibiting synthesis of maternal ^{67}Cu-ceruloplasmin with cycloheximide was investigated. Two pairs of pregnant rats were intravenously injected with ^{67}Cu (II) (on albumin + transcuprein). One pair was treated with cycloheximide 30 minutes before and 1.75 hours after the injection, and both pairs were sacrificed 4 hours after the injection. The results are shown in **Table A6.2(11)-4**. The cycloheximide-treated animals had much higher levels of ^{67}Cu in their livers and much less in the plasma, consistent with an inhibition of the synthesis of ^{67}Cu-ceruloplasmin by the maternal liver and its reduced release into the plasma. This was confirmed by chromatography (**Figure A6.2(11)-3**). Inhibition of ^{67}Cu-ceruloplasmin synthesis clearly reduced uptake of ^{67}Cu by placenta and foetal tissues, and a reduction in ^{67}Cu uptake was also observed for other maternal tissues. Uptake was half as great as otherwise in the case of placenta, and about one third as great in the foetus and foetal liver. Uptake was 25 – 50% reduced for maternal kidney, heart, spleen and brain. These findings confirm that ceruloplasmin is the most important source of copper for these tissues, and suggests that it might be the only source for some.
60.1.2 Copper in the foetal circulation.	The form of the copper appearing in foetal circulation after administration of ^{67}Cu to the dam was examined by gel chromatography and immunoprecipitation (**Figure A6.2(11)-4**). Independent of source, ^{67}Cu in the foetal plasma was found to be initially attached mainly to a component eluting in the void volume of Sephadex G-150 (**Figure A6.2(11)-4A**) and to a component eluting in the position of albumin (**α**-fetoprotein mainly substitutes for albumin in the foetus). A similar distribution of radioisotope was obtained when traces of ^{67}Cu-NTA were added to foetal serum in vitro before gel chromatography (**Figure A6.2(11)-4B**). On the same columns, ceruloplasmin oxidase activity from adult rat plasma eluted between the transcuprein and **α**-fetoprotein peaks (**Figure A6.2(11)-4B**). Evidence that the void volume peak was transcuprein was obtained by immunoprecipitation. Antibody raised against the ^{67}Cu-binding protein in the void volume peak from adult rat plasma was capable of precipitating ^{67}Cu from ^{67}Cu-transcuprein in adult rat plasma. This antibody also precipitated ^{67}Cu from the void volume peak of foetal plasma, but it failed to precipitate ^{67}Cu-albumin. Four hours after injection of copper radioisotope into the dams, ^{67}Cu in foetal plasma was still associated with transcuprein (**Figure A6.2(11)-4C**), but another peak had emerged in the position of ceruloplasmin. Assays of ceruloplasmin oxidase activity confirmed that the foetus had circulating ceruloplasmin. The level was only about one fifth that in the adult rats (mean ± SD, 3.2 ± 1.5 vs. 17.5 ± 2.4 units, with 1 unit being 10^{-5} IU/ml plasma). It rose to one third of adult values by day 2 after birth (5.2 ± 1.5 units). Pregnant rats had the same level of ceruloplasmin oxidase activity as their non-pregnant siblings (16.3 ± 31. vs. 17.5 ± units, respectively), and there were also traces of ceruloplasmin in the amniotic fluid.
60.1.3 Expression and translation of ceruloplasmin by maternal and foetal tissues.	The capacities of foetal and maternal tissue to produce ceruloplasmin were examined by assaying for expression of ceruloplasmin mRNA. Relative concentrations of the message were determined on subfractions of RNA associated with free polyribosomes, ER-bound polyribosomes, and that associated with the mRNP fraction. Equal portions of RNA

	from each fraction were slot-blotted onto nylon membranes and hybridised with [^{32}P]cDNA for rat ceruloplasmin. The bulk of the ceruloplasmin mRNA in the livers of non-pregnant (control) rats was found to be associated with the ER-bound polyribosomes, and that for tubulin was with the free polyribosomes. In maternal liver, most of the ceruloplasmin mRNA was also associated with the ER-bound polyribosomes. Some ceruloplasmin mRNA was also detected in the mRNP fraction. The same results were obtained for foetal liver. Indeed, the degree of hybridisation of the radioactive probe with equal amounts of RNA from maternal and foetal liver fractions was very similar, suggesting that both tissues were equally active in terms of ceruloplasmin protein synthesis and secretion. However, in placenta/yolk sac, mRNA for ceruloplasmin was even more abundant than in foetal and maternal liver.
60.1.4 Receptors for ceruloplasmin on placental membranes and membranes of other tissues	Placentae and other maternal and foetal tissues were probed for their content of membrane-associated ceruloplasmin receptors. Aliquots of placental microsomal membranes were incubated with various amounts of ^{67}Cu-ceruloplasmin, and the amount of radioactivity bound was determined. **Figure A6.2(11)-5** shows that binding appeared to obey saturation kinetics at low concentrations and that most of the binding could be diluted by the addition of a 100-fold excess of non-radioactive ceruloplasmin, indicative of specific binding. Binding was halfmaximal at about 0.5 nM. Total binding was also reduced in the presence of 300 µM Cu (II)-NTA. Total binding to placental membranes was about the same as that for maternal and virgin adult liver, and was reduced 50 ± 12% in the presence of excess Cu-NTA.
60.2 Discussion	The uptake of copper from the two major sources found in circulating blood of the rat in pregnancy were investigated. The results obtained indicated that ceruloplasmin was more effective than ionic copper (attached to albumin and transcuprein) in delivering this element to the foetus. This was apparent from the data for radioactivity expressed as a percentage of dose (per organ/tissue or per gram), in which ^{67}Cu from ceruloplasmin achieved a significantly higher concentration in the placenta and foetus, and accumulated much more rapidly with time. It was even more apparent when actual copper transfer was calculated, taking into account the sizes of copper pools ascribable to ceruloplasmin and albumin + transcuprein in the maternal circulation. For such calculations, it was assumed that ceruloplasmin and albumin+ transcuprein comprise 60% and 25% of copper plasma, respectively. On this basis, it was calculated that more than 200 ng of Cu were transferred to the foetuses (as a litter) from maternal plasma ceruloplasmin in 4 hours, as compared with less than 30 ng when Cu was given attached to albumin + transcuprein. This is a large difference. Indeed, since copper given as albumin and transcuprein rapidly disappeared from the circulation and was replaced by ceruloplasmin, even the ^{67}Cu injected as transcuprein + albumin may have entered placental tissue from ^{67}Cu-ceruloplasmin newly synthesised by the dam. This interpretation was confirmed by the finding that inhibition of endogenous ^{67}Cu-ceruloplasmin synthesis (from ^{67}Cu on albumin + transcuprein) reduced uptake of ^{67}Cu by almost all organs. The reduction was particularly strong in the case of the placenta and foetus, which emphasised the importance of ceruloplasmin for foetal uptake. It was also found that maternal organs (other than liver) depend largely on ceruloplasmin for copper.

Placental cells appeared to have membrane receptors for ceruloplasmin, and it is therefore likely that these are on the pathway taken by maternal ceruloplasmin copper to the foetus. The data suggest that transfer of copper from ceruloplasmin into the foetus is mainly a cell surface event not involving endocytosis, and that copper first enters the foetal circulation in ionic form, bound principally to nonceruloplasmin proteins, especially transcuprein. Evidence for the presence of transcuprein in foetal rat plasma is (i) that tracer ^{67}Cu is associated with a peak in the void volume of Sephadex G-150 columns; (ii) that this same peak appears when tracer ^{67}Cu(II) is added to foetal plasma *in vitro*; and (iii) that antibody raised against purified adult rat transcuprein precipitates ^{67}Cu associated with the foetal transcription peak. Albumin also appears to bind some of the copper in the foetal circulation, and **α**-fetoprotein is also likely to be involved.

This study has confirmed that the rat placenta has mRNA for ceruloplasmin. Furthermore, the message is being translated and the product secreted, as the message is associated with ER-bound polyribosomes. The data also suggest that foetal rat liver produces and secretes ceruloplasmin, the mRNA being associated with the ER-bound polyribosomes. Rat foetal liver is, therefore, likely to be a significant source of ceruloplasmin in the foetal circulation.

In conclusion, the findings of this study confirm the importance of ceruloplasmin in copper transport, not just for the normal mammalian adult, but also for the pregnant adult and the developing foetus.

61 APPLICANT'S SUMMARY AND CONCLUSION

61.1 Materials and methods

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

A study was conducted to investigate the tissue uptake of ^{67}Cu from ceruloplasmin versus that from albumin and transcuprein, after its intravenous administration to pregnant rats, in the last 4 days of gestation. For preparation of ^{67}Cu-labelled ceruloplasmin, a donor rat was injected with ^{67}Cu as neutralised nitrilotriacetate (NTA) complex. 12 to 19 hours later, the donor rat was sacrificed and plasma portions were fractionated on Sephadex G-150 columns. A single radioactive peak was obtained that gave a radioactive band in polyacrylamide gel electrophoresis characteristic of ceruloplasmin. The 3 most radioactive fractions were pooled for tail vein injection. For preparation of ^{67}Cu-labelled albumin + transcuprein, ^{67}Cu (labelled with ^{67}Cu(II)-NTA) was added to portions of rat plasma 2 – 50 hours before injection.

Radioactive samples were counted in a multisample gamma counter. Individual foetuses, foetal livers, placentae, and maternal organs were counted directly, as were samples of serum, column fractions, etc.

Sephadex G-150 chromatography was on 50 ml or 25 ml columns. Samples of 1.0 ml or 0.5 ml were applied to the larger and smaller columns, respectively. Fractions of 1.0 or 0.5 ml were collected for counting and other analyses.

Ceruloplasmin oxidase activity was measured using p-phenylene diamine as a substrate. In some cases, ceruloplasmin was identified by immunoprecipitation with specific rabbit anti-rat ceruloplasmin polyclonal antibody. Ceruloplasmin samples were also identified by their characteristic migration in disk gel electrophoresis on polyacrylamide.

		Receptor assays were carried out in the presence and absence of an excess of nonradioactive Cu (II) as NTA complex. Portions of membrane protein were incubated with ^{67}Cu-labelled ceruloplasmin for 1 hr at room temperature, before separation of bound and free ceruloplasmin by Airfuge. Total binding and binding in the presence of excess Cu-NTA were recorded. To demonstrate binding kinetics, portions of membrane from placenta were incubated with ^{67}Cu-ceruloplasmin in the absence and presence of excess non-radioactive ceruloplasmin, or "cold" Cu-NTA. Total ceruloplasmin mRNA was determined using extracts of total RNA obtained from portions of guanidine thiocyanate-treated tissue. The distribution of ceruloplasmin-mRNA to free and endoplasmic reticulum-bound polyribosomes was also examined. Postnuclear supernatants were fractionated on discontinuous sucrose gradients, and total RNA was extracted from the appropriate fractions. For mRNA determination, portions of RNA were slot-blotted and hybridised with [^{32}P]cDNA for rat ceruloplasmin. Densitometry of autoradiographs developed from the slot blots was performed with a spectrophotometer with a scanner. Control blots were made with [^{32}P]cDNA for ferritin and tubulin.
61.2	**Results and discussion**	*Summarize relevant results; discuss dose-response relationship.* ^{67}Cu infused as *in vivo*-labeled ceruloplasmin remained on ceruloplasmin in the maternal circulation over the 4- to 6-hr time period examined, as determined by gel chromatography and immunoreactivity. That infused as *in vitro*-labeled serum was initially on transcuprein and albumin but soon also appeared on new ceruloplasmin. By 4 hours post-injection, most ^{67}Cu was with ceruloplasmin. Production of this ceruloplasmin could be severely inhibited with cyclohexamide. On the basis of percent dose as well as total actual Cu transferred (taking into account the sizes of the two plasma Cu pools), ceruloplasmin was the preferred source of Cu for most tissues. Total uptake of Cu from ceruloplasmin was 7 times greater than that from albumin and transcuprein for the placenta, whole foetus, and foetal liver. It was 2- to 6-fold greater for other tissues (except liver and kidney). When synthesis of maternal ^{67}Cu-ceruloplasmin (from ^{67}Cu administered on albumin and transcuprein) was inhibited with cyctoheximide, uptake by nonhepatic tissues was reduced markedly. In the foetal circulation, incoming ^{67}Cu was initially associated with transcuprein and **α**-fetoprotein (or albumin), but also appeared within 4 hours with ceruloplasmin. Specific receptors for ceruloplasmin were detected on membranes from the placenta as well as foetal liver. mRNA for ceruloplasmin was detected on the endoplasmic reticulum-bound polyribosomes of placenta/yolk sac, and of foetal and maternal liver. This suggested that both tissues were active in terms of ceruloplasmin protein synthesis and secretion. It was concluded that Cu destined for the foetus is delivered mainly or exclusively by ceruloplasmin. It may enter via placental receptors, arriving in foetal plasma in ionic form, for later incorporation into foetal ceruloplasmin. The importance of ceruloplasmin as a source of plasma Cu for nonhepatic organs was also confirmed.
61.3	**Conclusion**	Copper that is transported to the liver is rapidly incorporated into ceruloplasmin, which is subsequently released into the systemic

	circulation for delivery to other tissues. Most tissues are able to take up copper from ceruloplasmin, with specific receptors having been demonstrated on liver and placenta cell surfaces.
61.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
61.3.2 Deficiencies	Yes. This study was not conducted and/or reported in compliance with GLP. When compared with generally accepted principles to be applied to toxicokinetics studies, as set out in OECD guideline 417, it is also apparent that methodological details were poorly reported in places, including: • Information on the animals from which samples of plasma were obtained for use in the study (eg. Numbers of animals used and the conditions in which they were housed); These deficiencies do not, however, necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes and that the results obtained are consistent with work published by other researchers. Furthermore, this research (including its methodology) was published in a peer-reviewed publication, 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. *(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]

Table A6.2(11)-2

Distribution of ^{67}Cu to Maternal and Foetal Tissues at Various Times after Intravenous ^{67}Cu-Ceruloplasmin

or ^{67}Cu (II) Attached to Albumin and Transcuprein[a]

Condition	^{67}Cu (% Total Dose/Tissue or Organ)				
	Maternal Plasma	Maternal Liver	Placenta	Foetal plasma	Foetal liver
After ^{67}Cu-ceruloplasmin					
1hr	74 ± 6 (8)	4.3 ± 0.3 (6)[b]	5.1 ± 1.4 (6)[b]	0.07± 0.38 (8)[b]	0.09 ± 0.14 (6)
2 hr	74 ± 3 (3)[b]	7.2 ± 0.4 (3)	4.5, 12.2 (2)	0.05± 0.01 (3)	0.50, 0.47 (2)
4 hr	73 ± 5 (8)[b]	6.1 ± 2.1 (5)	5.6 ± 1.8 (5)[b]	0.15 ± 0.08 (8)[b]	1.03 ± 0.43 (5)[b]
After ^{67}Cu(II)-labelled plasma					
1 hr	43 ± 30 (9)	10.4 ± 0.9 (8)	2.7 ± 0.7 (7)	0.04 ± 0.02 (9)	0.06 ± 0.04 (8)
2 hr	12 ± 5 (3)	9.1 ± 4.8 (3)	1.5, 1.3 (2)	0.02 ± 0.01 (3)	0.15, 0.13 (2)
4 hr	18 ± 8 (8)	10.4 ± 3.7 (5)	2.2 ± 0.9 (5)	0.02 ± 0.01 (8)	0.52 ± 0.15 (5)

[a] Total radioactivity recovered in each tissue or organ, assuming plasma is 5% of body weight. Data for placentae, foetal plasma, and liver were totals/litter. Values are ± SD for the number of litters or dams indicated in parentheses.
[b] P<0.01 for difference from rats with nonceruloplasmin 67Cu, determined by Student's *t* test.

Table A6.2(11)-4

Effect of Inhibiting Ceruloplasmin Formation with Cycloheximide on Tissue Deposition of Intravenous Injected ^{67}Cu(II) Given as Albumin Plus Transcuprein to Pregnant Rats[a]

Tissue	Total ^{67}Cu (% dose)		Organ wt (g)	
	-Cycloheximide	+Cycloheximide	-Cycloheximide	+Cycloheximide
Plasma	30, 35	10, 20	16.5, 14.8	17.3 15.7
Liver	31, 25	63, 50	10.6, 9.9	11.0, 9.8
Kidney	6.4, 5.1	3.5, 4.1	1.7, 1.5	1.9, 1.6
Spleen	0.30, 0.33	0.18, 0.15	0.6, 0.5	0.5, 0.3
Heart	0.42, 0.51	0.35, 0.25	0.9, 0.8	1.0, 0.9
Brain	0.15, 0.12	0.09, 0.08	1.7, 1.6	1.6, 1.7

Muscle	11.4, 13.4	9.6, 11.4	107, 100	110 103
Placentas	8.6, 9.7	4.0, 4.4	5.4, 5.2	6.2, 5.1
Foetuses	2.7, 2.3	0.6, 1.0	77, 61	68, 66
Foetal Livers	1.0, 0.9	0.3, 0.3	5.3, 4.6	4.8, 5.7
Foetal Plasma	0.12, 0.12	0.03, 0.5	3.8, 3.0	3.4, 3.2

- Pairs of pregnant rats were given intravenous ^{67}Cu(II) attached to albumin + transcuprein (as in Fig **A6.2(11)-1C**) by tail vein, 4 hr before sacrifice. Two rats were injected with cycloheximide intraperitoneally 30 min before and 1.75 hr after the intravenous ^{67}Cu to suppress formation of ^{67}Cu-ceruloplasmin. Data are values for radioactivity, as percentage of dose/organ for individual rats. Assumptions were plasma volume = 5% of body wt and muscle = 35% of body wt.

COMMENTS FROM ...

Date *Give date of comments submitted*

Table A6.2(11)-1

Body and Organ Weights (g) of Experimental Animals (mean ± SD)

	Body wt	Liver	Kidney	Spleen	Heart	Brain	Placenta	Uterus	Foetus	Foetal liver	No. in litter	Length (mm)
Pregnant rats												
After ^{67}Cu-ceruloplasmin												
1 hr (n=6)	344 ± 49	12.9 ± 0.6	1.7 ±0.1	0.9 ±0.2	1.0 ±0.1	1.7 ±0.2	0.47 ± 0.10	1.4, 0.9 (2)	3.3 ± 1.9	0.22 ± 0.13	12 ± 2	37 ± 8
4 h (n=5)	361 ± 43	12.7 ± 1.4	2.0 ±0.3	0.9 ±0.3	0.9 ±0.1	1.6 ±0.2	0.45 ± 0.07	1.1 ± 0.3	2.6 ± 1.0	0.18 ± 0.04	12 ± 2	36 ± 6
All times[a]	325 ± 91	13.1 ± 1.7	1.7 ±0.2	0.9 ±0.2	1.0 ±0.2	1.6 ±0.2	0.48 ± 0.08	1.1 ± 0.3 (8)	3.3 ± 1.6	0.24 ± 0.10	12 ± 2	36± 6
After ^{67}Cu-NTA												
1 h (n=8)	342 ± 21	12.0 ± 0.8	1.8 ±0.1	0.7 ±0.1	0.9 ±0.1	1.7 ±0.1	0.47 ± 0.06	1.5 ± 0.8 (5)	3.5 ± 1.3	0.22 ± 0.08	13 ± 2	37 ± 5
4 hr (n=5)	362 ± 21	11.1 ± 1.8	1.8 ±0.2	0.7 ±0.1	0.9 ±0.1	1.5 ±0.1	0.52 ± 0.08	1.0 ± 0.5	3.9 ± 1.6	0.22 ± 0.05	13 ± 2	37 ± 5
All times[a]	347 ± 26	12.0 ± 1.2	1.8 ±0.2	0.7 ±0.1	0.9 ±0.1	1.6 ±0.1	0.59 ± 0.26	1.2 ± 0.6	3.7 ± 1.5	0.23 ± 0.10	13 ± 2	36 ± 7
Nonpregnant rats												
All times (n=4)	291± 37	12.4 ± 4.0	2.5 ±0.6	0.8 ±0.3	1.1 ±0.3	1.8 ±0.2	--	--	--	--	--	--

Body and Organ Weights (g) of Experimental Animals (mean ± SD)

a Includes some rats sacrificed at 2 and 6 hr

Table A6.2(11)-2

Distribution of ^{67}Cu to Maternal and Foetal Tissues at Various Times after Intravenous ^{67}Cu-Ceruloplasmin or ^{67} Cu (II) Attached to Albumin and Transcuprein[a]

Condition	^{67} Cu (% Total Dose/Tissue or Organ)				
	Maternal Plasma	Maternal Liver	Placenta	Foetal plasma	Foetal liver
After ^{67}Cu-ceruloplasmin					
1hr	74 ± 6 (8)	4.3 ± 0.3 (6) [b]	5.1 ± 1.4 (6)[b]	0.07± 0.38 (8) [b]	0.09 ± 0.14 (6)
2 hr	74 ± 3 (3) [b]	7.2 ± 0.4 (3)	4.5, 12.2 (2)	0.05± 0.01 (3)	0.50, 0.47 (2)
After ^{67}Cu(II)-labelled plasma					
1 hr	43 ± 30 (9)	10.4 ± 0.9 (8)	2.7 ± 0.7 (7)	0.04 ± 0.02 (9)	0.06 ± 0.04 (8)
2 hr	12 ± 5 (3)	9.1 ± 4.8 (3)	1.5, 1.3 (2)	0.02 ± 0.01 (3)	0.15, 0.13 (2)
4 hr	18 ± 8 (8)	10.4 ± 3.7 (5)	2.2 ± 0.9 (5)	0.02 ± 0.01 (8)	0.52 ± 0.15 (5)

[a] Total radioactivity recovered in each tissue or organ, assuming plasma is 5% of body weight. Data for placentae, foetal plasma, and liver were totals/litter. Values are ± SD for the number of litters or dams indicated in parentheses.
[b] $P<0.01$ for difference from rats with nonceruloplasmin 67Cu, determined by Student's *t* test.

Table A6.2(11)-3

Uptake of ^{67}Cu from Ceruloplasmin or Other Plasma Proteins by Maternal Tissues and the Foetus, 1-4 Days before Term[a]

Condition	^{67}Cu uptake (% total dose)					
	Maternal Kidney	Maternal Spleen	Maternal Heart	Maternal Brain	Uterus	Whole Foetuses
After ^{67}Cu-ceruloplasmin						
1 hr (6)	1.2 ± 0.4	0.24 ± 0.07	0.53 ± 0.11[b]	0.14 ± 0.04[b]	1.0 ± 0.8 (3)	0.15± 0.05
2 hr (3)	2.0 ± 0.2	0.31 ± 0.05[b]	0.58 ± 0.21	0.12 ± 0.11	0.43, 0.45 (2)	0.71, 0.52 (2)
4 hr (5)	2.2 ± 1.3	0.24 ± 0.05	0.54 ± 0.16[b]	0.10 ± 0.08	0.69± 0.24	1.57± 0.40[b]
After ^{67}Cu (II)-labelled plasma						
1 hr (6)	2.5 ± 0.8	0.19 ± 0.04	0.17 ± 0.07	0.05 ± 0.03	1.0 ± 0.7 (5)	0.18 ± 0.14
2 hr (3)	5.9 ± 3.7	0.12 ± 0.04	0.14 ± 0.06	0.03 ± 0.01	0.57, 0.47 (2)	0.12, 0.18 (2)
4 hr (5)	2.4 ± 0.7 (4)	0.22 ± 0.07	0.24 ± 0.13	0.04 ± 0.02	0.68 ± 0.46	0.52 ± 0.15

a Values are mean ±SD for the numbers indicated. Data were obtained as in Table A.6.2.7-2 and are for the same animals.
b $P<0.01$ for difference from rats infused with nonceruloplasmin – ^{67}Cu, determined by Student's *t* test.

Table A6.2(11)-4

Effect of Inhibiting Ceruloplasmin Formation with Cycloheximide on Tissue Deposition of Intravenous Injected ^{67}Cu(II) Given as Albumin Plus Transcuprein to Pregnant Rats[a]

Tissue	Total ^{67}Cu (% dose)		Organ wt (g)	
	-Cycloheximide	+Cycloheximide	-Cycloheximide	+Cycloheximide
Plasma	30, 35	10, 20	16.5, 14.8	17.3 15.7
Liver	31, 25	63, 50	10.6, 9.9	11.0, 9.8
Kidney	6.4, 5.1	3.5, 4.1	1.7, 1.5	1.9, 1.6
Spleen	0.30, 0.33	0.18, 0.15	0.6, 0.5	0.5, 0.3
Heart	0.42, 0.51	0.35, 0.25	0.9, 0.8	1.0, 0.9
Brain	0.15, 0.12	0.09, 0.08	1.7, 1.6	1.6, 1.7
Muscle	11.4, 13.4	9.6, 11.4	107, 100	110. 103
Placentas	8.6, 9.7	4.0, 4.4	5.4, 5.2	6.2, 5.1
Foetuses	2.7, 2.3	0.6, 1.0	77, 61	68, 66
Foetal Livers	1.0, 0.9	0.3, 0.3	5.3, 4.6	4.8, 5.7
Foetal Plasma	0.12, 0.12	0.03, 0.5	3.8, 3.0	3.4, 3.2

[a] Pairs of pregnant rats were given intravenous ^{67}Cu(II) attached to albumin + transcuprein (as in Fig ?) by tail vein, 4 hr before sacrifice. Two rats were injected with cycloheximide intraperitoneally 30 min before and 1.75 hr after the intravenous ^{67}Cu to suppress formation of ^{67}Cu-ceruloplasmin. Data are values for radioactivity, as percentage of dose/organ for individual rats. Assumptions were plasma volume = 5% of body wt and muscle = 35% of body wt.

Figure A6.2(11)-1

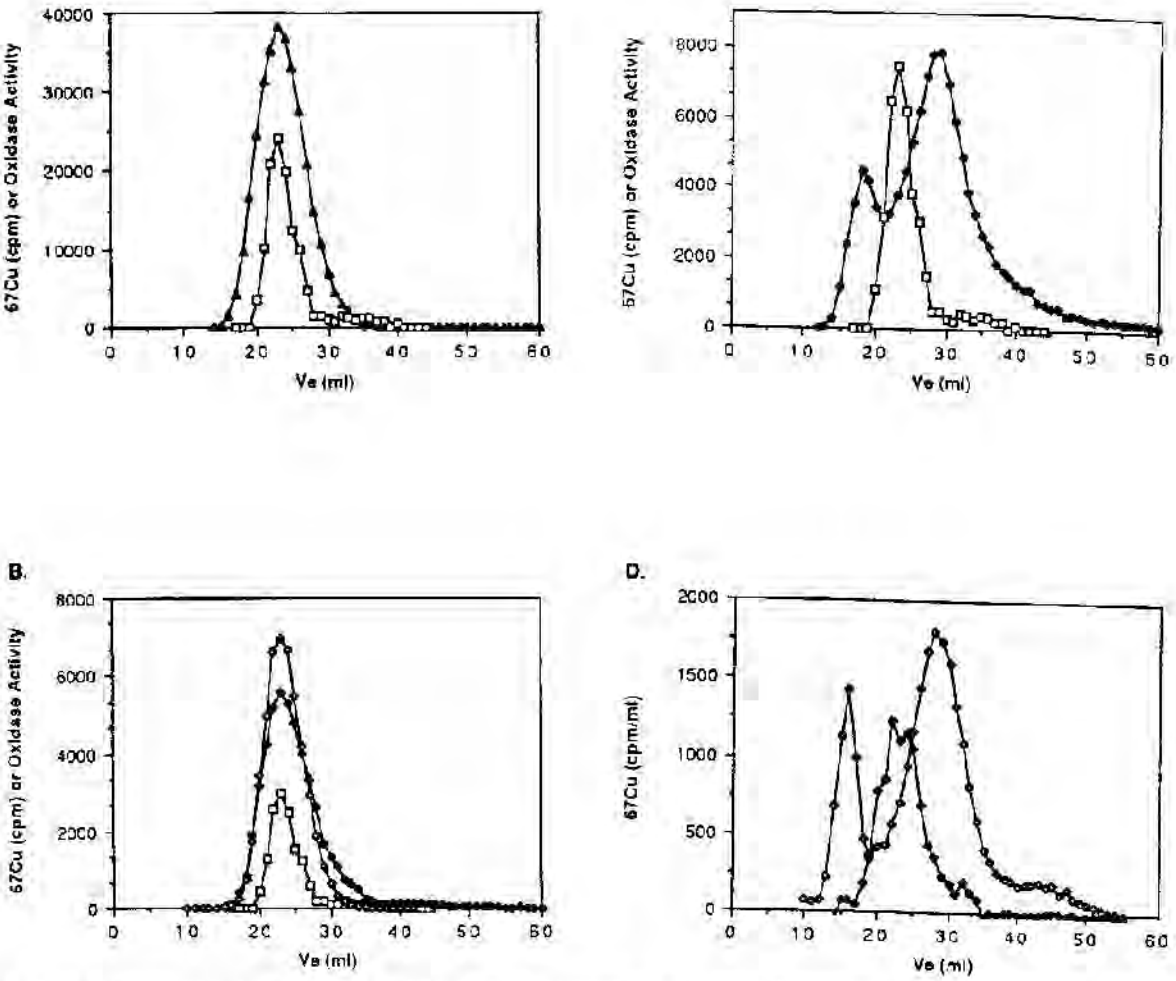


Figure 1. Gel chromatography of ^{67}Cu-labeled samples used for intravenous infusion, and serum samples from the maternal circulation taken 1 and 4 hr after infusion. One-milliliter samples were chromatographed on 50-ml columns of Sephadex G-150. Ceruloplasmin and albumin standards eluted at 23 ml and 28 ml in these columns, respectively. (A) An example of the partially purified ^{67}Cu-ceruloplasmin used in the intravenous infusions, showing elution of radioactivity (▲) in relation to ceruloplasmin oxidase activity (□). (B) Maternal serum at 1 hr (◇) and 4 hr (◆) after tail vein infusion of the ^{67}Cu-ceruloplasmin in (A). Elution of ceruloplasmin oxidase activity is also shown (□). (C) Nonradioactive rat serum to which 2 ng of Cu (as ^{67}Cu(II)-NTA) had been added *in vitro* (◆), which was infused as ^{67}Cu attached to albumin + transcuprein. (The comparative elution of ceruloplasmin oxidase activity is shown again.) (D) Maternal serum at 1 hr (◇) and 4 hr (◆) after infusion of albumin + transcuprein-bound ^{67}Cu (mixture shown in [C]). For the 4-hr sample, cpm values have been multiplied by two to make them more visible.

Figure A6.2(11)-2

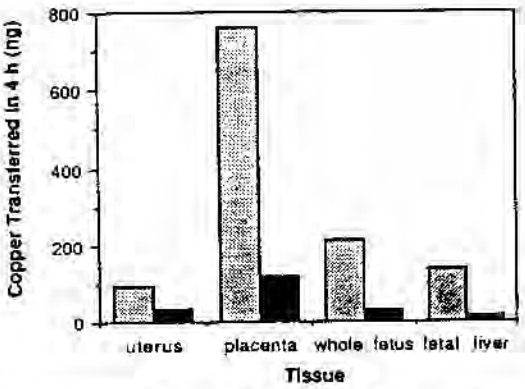


Figure 2. Estimates of actual nanograms of Cu transferred from the maternal circulation to pregnancy and fetal tissues over 4 hr, from ceruloplasmin (shaded bars) and from albumin + transcuprein (closed bars). Mean values for percentage of dose of radioactivity per organ (in Table II) and sizes of plasma copper pools (in ng Cu/ml) were used to calculate the specific activity of ^{67}Cu in ceruloplasmin as well as in albumin + transcuprein (as percentage of dose per ng Cu) after injection of the radioactive samples into the plasma. Assumptions used were plasma volume = 5% of body wt; body wt = 360 g; ceruloplasmin Cu = 750 ng/ml of plasma, totaling 13.5 μg/whole animal; albumin + transcuprein Cu = 300 ng/ml, totaling 5.4 μg/animal. Less than 50-ng portions of Cu were injected as ceruloplasmin or transcuprein + albumin, a negligible amount in relation to the sizes of the inherent copper pools labeled by the radioisotope. The values shown are mean percentage of dose × pool size, in nanograms. Values for placenta and uterus represent amounts retained at the 4-hr time point (i.e., they do not take into account any copper transferred to the fetus).

Figure A6.2(11)-3

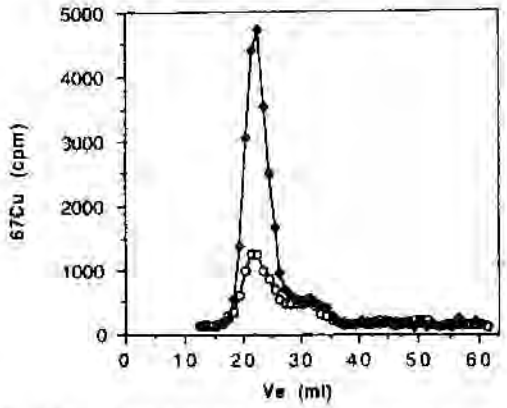


Figure 3. Effect of cycloheximide treatment on production of ^{67}Cu-ceruloplasmin by maternal tissues. Samples of plasma from pregnant rats injected intravenously with equal doses of ^{67}Cu(II) on albumin + transcuprein 4 hr before, were fractionated on Sephadex G-150, as in Figure 1. One rat received cycloheximide before and during the ^{67}Cu uptake period (○), the other did not (●). (Similar results were obtained for another pair of rats.) Samples were run on the same column and decay-corrected to the same time.

Figure A6.2(11)-4

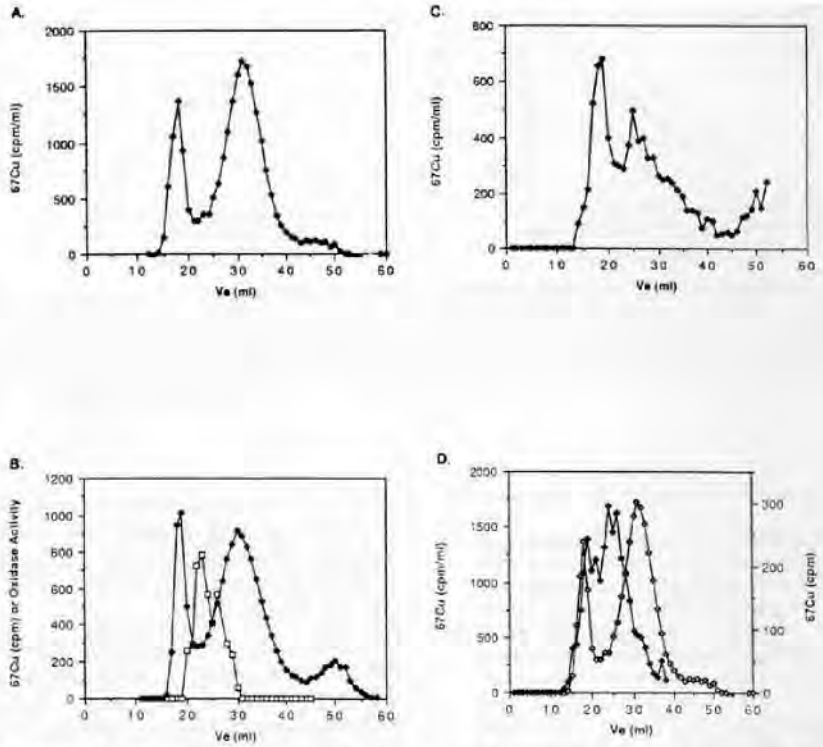


Figure 4. (A) Chromatography of fetal plasma samples taken at different times after infusion of ^{67}Cu components into the maternal circulation (as in Fig. 1). Fetal plasma from rats sacrificed 1 hr after infusion of ^{67}Cu-ceruloplasmin. (The same kind of chromatograph was obtained when ^{67}Cu-labeled transcuprein + albumin was infused.) (B) Fractionation of 1.0 ml of fetal plasma labeled *in vitro* with 2 ng of ^{67}Cu(II)-NTA. The elution position of ceruloplasmin oxidase activity (□) is also indicated. (C) ^{67}Cu-labeled components in fetal plasma 4 hr after maternal infusion of ^{67}Cu-ceruloplasmin. (The same kind of chromatograph was obtained after infusion of ^{67}Cu-labeled transcuprein + albumin.) (D) Another profile of ^{67}Cu-binding components in fetal plasma (♦) less commonly obtained 1 hr after infusion of ^{67}Cu (on albumin + transcuprein) into the mother, in comparison with the more usual profile (○) also shown in (A).

Figure A6.2(11)-5

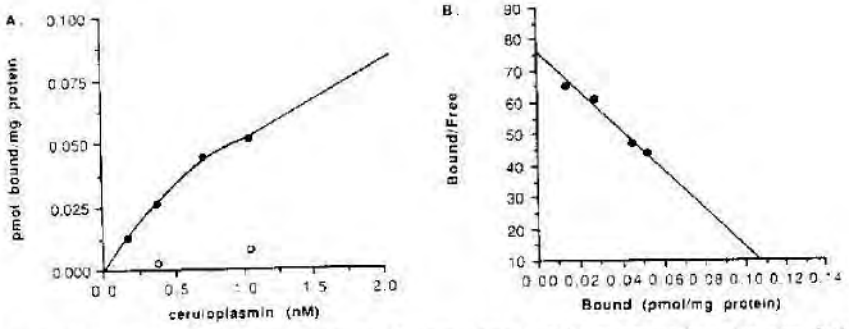


Figure 6. Binding of ^{67}Cu-ceruloplasmin to placental membranes. Aliquots of microsomal membranes from placentae of rats 1 day before term were incubated with various concentrations of ^{67}Cu-ceruloplasmin, partially purified from the plasma of a donor rat, for 2 hr at 25°C, before separation of free and bound radioactivity by ultracentrifugation. (A) Total binding of radioactivity (●); binding in the presence of a 100-fold excess of nonradioactive ceruloplasmin was tested at some concentrations (○). Not shown is binding that was measured at much higher concentrations (up to 200 n*M*), which was only partially (30–50%) inhibited by excess "cold" ceruloplasmin (due to nonspecific binding). (B) Data from (A) plotted according to Scatchard (54).

			Official use only
		62 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).* Wirth, W.L. and Linder, M.M. (1985). Distribution of Copper Among Components of Human Serum. JNCI. **75**: 277-284 (published).	X
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	
		63 GUIDELINES AND QUALITY ASSURANCE	
63.1	**Guideline study**	No. This was a non-regulatory study carried out to determine the distribution of copper among components of human plasma, by the used of highly sensitive furnace atomic absorption spectroscopy. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
63.2	**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)*	
63.3	**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")*	
		64 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.*	
64.1	**Test material**	Samples of human sera were analysed for copper content.	
64.1.1	Lot/Batch number	Not applicable	
64.1.2	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 5.0/12	**A6.2(12), Distribution of copper**

64.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Frozen samples (1 – 3 ml) of leftover sera from patients with a variety of different types of cancer were obtained from the clinical laboratory of Anaheim Memorial hospital by permission of the hospital staff and ethics committee. 44 other frozen samples were obtained from the NCI-Mayo Clinic Serum Bank. These were from men and women, aged 45 to 75 years, with colon cancer, ulcers or polyps, or no disease. Samples of normal sera were also collected from volunteers at the testing laboratory. All samples were kept frozen at –20°C for up to 6 months.	
64.1.2.2 Purity	*Give purity in % of active substance* [redacted]	
64.1.2.3 Stability	*Describe stability of test material* There was no loss in ceruloplasmin oxidase activity of samples stored frozen and then thawed up to 4 times over a period of up to 1 year.	
64.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* Not necessary for a study of this type.	
64.2 Test Subjects	*Non*-entry *field*	
64.2.1 Species	Human	
64.2.2 Strain	Not applicable	
64.2.3 Source	See section 2.1.2.1	X
64.2.4 Sex	Male and female	
64.3 Procedures	Non-entry field	
64.3.1 Cu analysis and oxidase assays	The total Cu content of serum samples and serum fractions was determined by furnace atomic absorption spectroscopy using a model 475 spectrometer, with or without prior wet ashing. For wet ashing, 0.10 ml samples were digested at 300 – 500°C with 0.50 ml of a mixture of ultrapure nitric, sulphuric, and perchloric acids (24:24:1). The digested residue was dissolved in 10 mM ultrapure sulphuric acid for furnace atomic absorption analysis (sensitivity in the 10 – 30 ppb range, with backgrounds of 0 – 10 ng/ml (ppb)). The p-phenylenediamine oxidase activity of ceruloplasmin was assayed by use of 50 or 100 µl serum samples and larger samples of fractionated serum. The method followed spectrophotometrically (at 540 nm) the oxidation of p-phenylenediamine to a purple product (Bandrowski's base), at 37°C, pH 5.5, in acetate buffer with ethylenediaminetetraacetic acid (EDTA); 10 mM) added to prevent nonspecific oxidation of substrate by traces of free iron. Enzyme activity was reported in terms of 10^{-5} IU ceruloplasmin (activity)/ml.	
64.3.2 Chromatography	Gel chromatography of serum samples was performed on 50 ml columns of Sephadex G-150, pretreated with sodium borohydride to reduce metal adsorption. Columns were equilibrated with 0.15 N NaCl or 20 mM potassium phosphate (pH 7.0). Samples at 1.0 ml were applied and eluted with the same reagent. Fractions of 0.9 - 1.0 or 1.1 ml were collected and assayed for Cu content or other substituents. Affinity chromatography was performed with 10 ml columns of Affi-gel blue. Columns were equilibrated with 20 mM potassium phosphate	

buffer (pH 7.0). Serum samples (1.5 – 2.0 ml) diluted 1:5 with the same buffer were applied to the column. After elution of almost all material absorbing at 280 nm, the same buffer but containing 2 M NaCl was applied to elute the albumin plus transcuprein fractions. Final stripping of the column was with 0.5 M KSCN (in the same buffer). Fractions of 2 – 5 ml were collected and analysed.

65 RESULTS AND DISCUSSION

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

65.1 Results

Non-entry field.

65.1.1 Profiling Serum Cu Components: Methodology

When samples of human sera (1.0 ml) were fractionated on 50 ml columns of Sephadex G-150 and the samples analysed for Cu content by furnace atomic absorption spectrometry, one major and three or more smaller peaks or shoulders were apparent (**Figure A6.2(12)-1**). In order of their elution, these peaks contained transcuprein (apparent mw 270,000); ceruloplasmin (mw 132,000), the major peak, coinciding with ceruloplasmin oxidase activity; and albumin (mw 68,000). Also, 1 – 3 small peaks of lower molecular weight were also found within the column volume. From the profile shown in **Figure A6.2(12)-1**, the ng of Cu recovered in transcuprein (fractions 15-18), ceruloplasmin (fractions 19-25), and albumin (fractions 26-30) and in components of low molecular weight (fractions 31-60) were determined by adding up the Cu (minus background) in the relevant fractions. The total in each peak was also compared with the total recovered on the column. Values, in this case, were either 230, 710, 170 and 90 ng from a total of 1119 ng, or 19, 60, 14 and 7% for the transcuprein, ceruloplasmin, albumin and low molecular weight Cu fractions, respectively. With comparisons of the total recovered in column fractions and the total applied (determined by direct analysis of diluted serum), average recoveries were 94 ± 21% SD (n = 16) and 106 ± 32% (n = 21).

The least satisfactory separation on sephadex G-150 was between ceruloplasmin and albumin-bound Cu. Affinity chromatography on Affi-gel blue was therefore used to further clarify the distribution of copper between these components. As shown in **Figure A6.2(12)-2**, the proportions of Cu found in ceruloplasmin vs. other components were very similar to those estimated from the Sephadex profiles for the same sample (**Table A6.2(12)-1**).

The possibility that the furnace technique was not fully analysing the Cu present and that prior wet ashing was necessary was also assessed. Column fractions for each of the components was combined, and aliquots of the pooled material were tested with and without prior wet ashing. The results indicated that prior wet ashing made no significant difference in values obtained for any of the Cu components. Specifically, values for non-wet-ashed samples averaged 101% of those for wet-ashed samples (SD = 30; n = 11).

65.1.2 Profiling Serum Cu Components for Normal People and People With Cancer.

Two studies were undertaken using the Sephadex G-150 profiling technique to determine the distribution of Cu among plasma components of normal subjects and those with cancer.

In the first study, normal samples came from volunteers of both sexes within the testing laboratory, whereas patient samples were from individuals with a variety of different forms of cancer. The results are

shown in **Table A6.2(12)-2**. There was considerable variation in the relative copper contents of the four serum components, within both the normal group and those with diverse forms of cancer. Patients with cancer tended to have greater concentrations of serum Cu than did normal subjects. However, the overall averages for these groups demonstrated that ceruloplasmin was the major component, with about two-thirds of the Cu present in normal serum and slightly less in cancer patients. The transcuprein and albumin fractions had approximately equal proportions of Cu, and there was a variable amount detectable in the low-molecular-weight region, averaging less than 10%.

For the second study, samples were obtained from patients with colon cancer at various stages, from controls with non-malignant intestinal tract diseases (ulcers or polyps), and from normal subjects. All groups included samples from both men and women. The results are shown in **Table A6.2(12)-3**. The proportions of Cu seen in the various components were similar to those seen in the first study, with the possible exception of Cu in albumin and low molecular weight components. None of the differences between data in **Table A6.2(12)-2** and **Table A6.2(12)-3** achieved statistical significance ($P<0.05$)). For the colon cancer group, total Cu was increased an average of 50% and a significant portion of this increase came from ceruloplasmin Cu. Mean values for albumin and transcuprein were also greater than the mean value for the normal group, but did not achieve statistical significance. In general, the variability of values for each component was greater in the two diseased groups than it was for the normal subjects. The mean values suggested that, on average, in cancer patients all 4 components were increased over the normal in terms of their Cu contents.

The consistency of the elution volumes in the column runs and the consistent appearance of specific Cu peaks in the low-molecular-weight region of the columns are indicated in **Table A6.2(12)-4** for 22 columns. On average, the transcuprein fraction had a peak elution volume of 17 ml; ceruloplasmin , 25 ml; and albumin 30 ml. Three components of low molecular weight, eluting at 39, 47 and 50 ml, were present in a large proportion of the serum samples. This region of the elution profile corresponded to molecular weights in the range 13,000 to 30,000, as determined from a series of low molecular weight standards (**Figure A6.2(12)-3**). In analyses done beyond the 50 ml volume, a peak was also often present at 60 ml. This finding is considered to represent Cu complexes with amino acids.

65.2 Discussion

The results of this study have shown that normal human serum contains about 1 µg Cu/ml; about two thirds of this is associated with ceruloplasmin; about 15% each is associated with the albumin and transcuprein fractions; the rest (about 10%) is associated with low molecular weight components (small proteins). Additional Cu eluted beyond the column volume may represent complexes with amino acids.

Within the normal groups, a few people had levels of Cu much higher than average in the transcuprein or albumin fractions and/or in certain low molecular weight components. Ceruloplasmin levels tended to be stable, except in disease. In the cancer patients, the proportions of Cu distributed to the 4 compartments were, in general, similar to the proportion for the normal group, although there was greater variability. Average total serum Cu was increased, although many individuals were in the normal range. On average, significantly more Cu was associated with ceruloplasmin in the sera from the cancer patients; also, Cu tended

to be more associated with the transcuprein and albumin fractions.

66 APPLICANT'S SUMMARY AND CONCLUSION

66.1 Materials and methods

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

A non-regulatory, non-guideline, study was carried out to determine the distribution of copper among different components of human plasma.

Samples of sera were obtained from normal volunteers and from patients with a variety of different types of cancer, including colon cancer. Samples were also collected from individuals suffering from colonic ulcers or polyps. All samples were kept frozen at –20°C.

The total Cu content of serum samples and serum fractions was determined by furnace atomic absorption spectroscopy (FAAS), with or without prior wet ashing. For wet ashing, 0.10 ml samples were digested at 300 – 500°C with 0.50 ml of a mixture of nitric, sulphuric, and perchloric acids (24:24:1). Digested residues were dissolved in 10 mM sulphuric acid for FAAS analysis (sensitivity in the 10 – 30 ppb range, with backgrounds of 0 – 10 ng/ml (ppb)).

The p-phenylenediamine oxidase activity of ceruloplasmin was assayed by spectrophotometrically following (at 540 nm) the oxidation of p-phenylenediamine to a purple product (Bandrowski's base), at 37°C, pH 5.5, in acetate buffer. 10 mM EDTA was added to prevent nonspecific oxidation of substrate by traces of free iron. Enzyme activity was reported in terms of 10^{-5} IU ceruloplasmin (activity)/ml.

Gel chromatography of serum samples was performed on 50 ml columns of Sephadex G-150, pretreated with sodium borohydride to reduce metal adsorption. Columns were equilibrated with 0.15 N NaCl or 20 mM potassium phosphate (pH 7.0). 1.0 ml samples were applied and eluted with the same reagent. 0.9 - 1.0 ml or 1.1 ml fractions were collected and assayed by FAAS for Cu content or other substituents.

Affinity chromatography was performed with 10 ml columns of Affi-gel blue. Columns were equilibrated with 20 mM potassium phosphate buffer (pH 7.0). Serum samples (1.5 – 2.0 ml) diluted 1:5 with the same buffer were applied to the column. After elution of almost all material absorbing at 280 nm, the same buffer but containing 2 M NaCl was applied to elute the albumin plus transcuprein fractions. Final stripping of the column was with 0.5 M KSCN (in the same buffer). Fractions of 2 – 5 ml were collected and analysed by FAAS.

66.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

When samples of human sera were fractionated columns of Sephadex G-150 and the samples analysed for Cu content by FAAS, one major and 3 or more smaller peaks or shoulders were apparent. In order of elution, these peaks contained transcuprein, ceruloplasmin and albumin. 1–3 small peaks of lower molecular weight were also found within the column volume. The amount of Cu recovered in transcuprein, ceruloplasmin, albumin and low molecular weight components were 230, 710, 170 and 90 ng respectively from a total of 1119 ng (i.e. 19, 60, 14 and 7%, respectively). Evaluation of the total Cu recovered in column fractions and the total applied (determined by direct analysis of diluted serum) gave average recoveries of 94 ± 21% SD (n = 16) and 106 ± 32% (n = 21), respectively. Affinity chromatography on Affi-gel

blue was used to confirm the distribution of copper between these components. The proportions of Cu found in ceruloplasmin vs. other components were similar to those estimated from the Sephadex profiles.

Two studies were undertaken using the Sephadex G-150 profiling technique to determine the distribution of Cu among plasma components of normal subjects and those with cancer.

In the first study, normal samples came from volunteers of both sexes within the testing laboratory, whereas patient samples were from individuals with a variety of different forms of cancer. There was considerable variation in the relative copper contents of the four serum components, within both the normal group and those with cancer. Patients with cancer tended to have greater concentrations of serum Cu than did normal subjects. However, overall averages for these groups demonstrated that ceruloplasmin was the major component, with about two-thirds of Cu present in normal serum and slightly less in cancer patients. Transcuprein and albumin fractions had approximately equal proportions of Cu, and there was a variable amount detectable in the low molecular-weight region, averaging less than 10%.

For the second study, samples were obtained from patients with colon cancer at various stages, from controls with non-malignant intestinal tract diseases (ulcers or polyps), and from normal subjects. All groups included samples from both men and women. The proportions of Cu seen in the various components were similar to those seen in the first study, with the possible exception of Cu in albumin and low molecular weight components. For the colon cancer group, total Cu was increased an average of 50% and a significant portion of this increase came from ceruloplasmin Cu. Mean values for albumin and transcuprein were also greater than the mean value for the normal group, but did not achieve statistical significance. In general, the variability of values for each component was greater in the two diseased groups than it was for the normal subjects. The mean values suggested that, on average, in cancer patients all 4 components were increased over the normal in terms of their Cu contents.

66.3 Conclusion

The results of this study have shown that normal human serum contains about 1 µg Cu/ml; about two thirds of this is associated with ceruloplasmin; about 15% each is associated with the albumin and transcuprein fractions; the rest (about 10%) is associated with low molecular weight components (small proteins). Additional Cu eluted beyond the column volume may represent complexes with amino acids.

66.3.1 Reliability

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

2

66.3.2 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, the study has been referenced on a number of occasions in expert reviews of copper toxicokinetics.

No internationally accepted guidelines are available that specifically

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.)

Evaluation by Competent Authorities
[redacted]
[redacted]

Table A6.2(12)-3

Cu components in serum of colon cancer patients and controls

Variables	Normal subjects[a]	Colon cancer patients [a, b]	Patients with nonmalignant gastrointestinal disease [a]
Age, yr	61± 13 (14)	65± 8 (16)	61± 14 (14)
Sex, No. of male/No. of female	7/7	6/10	8/6
Ceruloplasmin oxidase activity, [c] 10^5 IU/ml	16± 3 (7)	31± 6 (8)[d]	21± 2 (7)
Total serum, Cu, ng/ml	1,030 ± 130 (7)	1,520 ± 330 (8)[d]	1,250 ± 390 (7)
Transcuprein fraction	120 ± 30	160 ± 70	170 ± 110
Ceruloplasmin	600 ± 90	950 ± 210[d]	640 ± 330
Albumin fraction	170 ± 70	210 ± 60	150 ± 130
Low mol wt	110 ± 40	110 ± 60	140± 110
Percent of total Cu			
Transcuprein fraction	12 ± 2	11 ± 4	11± 11
Ceruloplasmin	61 ± 4	64 ± 10	57± 3
Albumin fraction	16 ± 5	12 ± 4	16± 6
Low mol wt	12 ± 5	8 ± 3	11± 4 (5)
			[27± 32 (7)] [e]

[a] Means ± SD (No.).
[b] All but 4 of the patients had metastases; half had metastases at distant sites.
[c] Mean ± Sd values are calculated for average values of pairs of serum samples pooled for each "profiling" analysis. Pooling of samples with similar ceruloplasmin oxidase activities was necessary to have enough serum for the column runs.
[d] $P<0.01$ for difference from value for normal subjects.
[e] *Values in brackets* indicate mean ± SD for all 7 samples.

Table A6.2(12)-4

Elution Volume of serum Cu components for samples from patients with colon cancer and controls.

Sample	Elution vol, ml[a]					
	Transcuprein fraction	Ceruloplasmin	Albumin fraction	Low-mol-wt components		
Normal	18	27	31	40	45	(50)
	15	23	27	31 (37)	49	-
	20	28	31	-	-	(50)
	17	25	31	40*	47*	51*
	17	23	30	-	-	-
	17	22	28	(40)	49	-
	18	24	30	-	-	50
Nonmalignant gastro-Intestinal disease	17	22	28	40	49	-
	18	24	30	-	-	50
	16	24	31	40*	48	50*
	17	26	31	42	-	50*
	17	26	31	40	44	-
	18	26	31	-	45*	-
	17*	24	29	-	-	-

Colon cancer	18*	25	31	40	45	-
	16	24	30	-	-	50
	18	24	30	36 (40)	-	-
	17	24	28	38	-	-
	18	27	31	39	48*	52
	18	26	30	40	43,45	50
	16	22	26	39	(48)	-
	19	26	32	40*	45*	-
Overall, mean ± SD	17 ± 1	25 ± 2	30 ± 2	39 ± 1	47 ± 2	50± 1
	(22)	(22)	(22)	(15)	(13)	(10)

[a] *Numbers in parentheses* refer to small peaks; *dash* indicates no peak; *asterisk* indicates pronounced, very large peak.

COMMENTS FROM ...

Date *Give date of comments submitted*

Table A6.2(12)-1

Comparison of serum fractionations by Sephadex G-150 and Affi-gel blue[a]

Sample No.	Sample per method	Total serum Cu, ng/ml	Percentage in: Ceruloplasmin faction	Transcuprein fraction	Albumin fraction	Low-mol-wt components
I	Sephadex G-150	1,050	74	11	10	5
	Affi-gel blue		70	25		6
II	Sephadex G-150	910	77, 73	9, 7	15, 18	7, 9
	Affi-gel blue		76, 65	15, 21		
III	Sephadex G-150	950	79, 79	13, 7	6, 9	2, 5
	Affi-gel blue		77	16		7

[a] Results of individual determinations (column runs) on 3 different samples.

Table A6.2(12)-2

Distribution of Cu among serum components in normal subjects and in patients with different kinds of cancer.

Sample	Serum fraction: Total	Transcuprein	Ceruloplasmin	Albmin	Low-mol-wt Cu
	ng Cu/component/ml				
Normal subjects,[a] mean ± SD (6)	850 ± 200	120±30	570 ± 100	120 ± 40	70±60
Type of cancer[b]					
Gastric	1,050	400	540	70	40
Gastric	1,320	540	430	240	110
Colon (metast)	950	200	480	230	40
Colon (metast)	3,350	610	1,550	970	310
Breast	710	40	520	150	10
Breast (metast)	940	110	630	200	0
Breast (metast)	1, 130	100	840	110	80
Bronchogenic	760	120	450	180	20
Misc. (metast)	1,200	60	930	210	10
Misc. (metast)	1,550	200	1,040	230	80
Misc. (metast)	1,080	220	570	280	10
All cancer patients,[c] mean ± SD (11)	1,290±730	300±200	740±350	260±240	40±40
	Percent of total serum Cu				
Normal subjects		14±2	65±11	14±4	8±5
Cancer subjects		18±11	55±13	19±7	4±3

[a] Age, 24±3 yr (mean ± SD): 4 women, 2 men.
[b] Misc. = miscellaneous; metast = metastases present.
[c] Cancer patients ranged in age from 53 to 89 yr (average, 71±11; mean ± SD) and 7 of 11 were female.

Table A6.2(12)-3

Cu components in serum of colon cancer patients and controls

Variables	Normal subjects[a]	Colon cancer patients [a, b]	Patients with nonmalignant gastrointestinal disease [a]
Age, yr	61± 13 (14)	65± 8 (16)	61± 14 (14)
Sex, No. of male/No. of female	7/7	6/10	8/6
Ceruloplasmin oxidase activity, [c] 10^5 IU/ml	16± 3 (7)	31± 6 (8)[d]	21± 2 (7)
Total serum, Cu, ng/ml	1,030 ± 130 (7)	1,520 ± 330 (8)[d]	1,250 ± 390 (7)
Transcuprein fraction	120 ± 30	160 ± 70	170 ± 110
Ceruloplasmin	600 ± 90	950 ± 210	640 ± 330
Albumin fraction	170 ± 70	210 ± 60	150 ± 130
Low mol wt	110 ± 40	110 ± 60	140± 110
Percent of total Cu			
Transcuprein fraction	12 ± 2	11 ± 4	11± 11
Ceruloplasmin	61 ± 4	64 ± 10	57± 3
Albumin fraction	16 ± 5	12 ± 4	16± 6
Low mol wt	12 ± 5	8 ± 3	11± 4 (5)
			[27± 32 (7)] [e]

[a] Means ± SD (No.).
[b] All but 4 of the patients had metastases; half had metastases at distant sites.
[c] Mean ± Sd values are calculated for average values of pairs of serum samples pooled for each "profiling" analysis. Pooling of samples with similar ceruloplasmin oxidase activities was necessary to have enough serum for the column runs.
[d] $P<0.01$ for difference from value for normal subjects.
[e] *Values in brackets* indicate mean ± SD for all 7 samples.

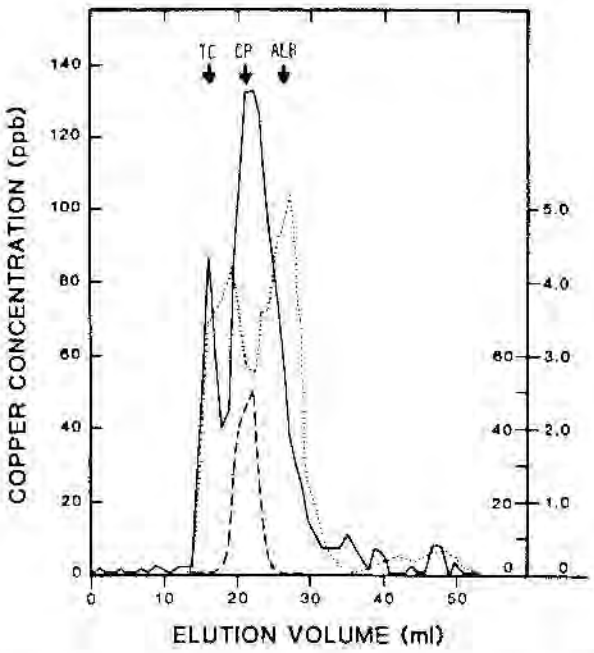
Table A6.2(12)-4

Elution Volume of serum Cu components for samples from patients with colon cancer and controls.

Sample	Elution vol, ml[a]					
	Transcuprein fraction	Ceruloplasmin	Albumin fraction	Low-mol-wt components		
Normal	18	27	31	40	45	(50)
	15	23	27	31 (37)	49	-
	20	28	31	-	-	51*
	17	25	31	40*	47*	51*
	17	23	30	-	-	-
	17	22	28	(40)	49	-
	18	24	30	-	-	50
Nonmalignant gastro-Intestinal disease	17	22	28	40	49	-
	18	24	30	-	-	50
	16	24	31	40*	48	50*
	17	26	31	42	-	50*
	17	26	31	40	44	-
	18	26	31	-	45*	-
	17*	24	29	-	-	-
Colon cancer	18*	25	31	40	45	-
	16	24	30	-	-	50
	18	24	30	36 (40)	-	-
	17	24	28	38	-	-
	18	27	31	39	48*	52
	18	26	30	40	43,45	50
	16	22	26	39	(48)	-
	19	26	32	40*	45*	-
Overall, mean ± SD	17 ± 1	25 ± 2	30 ± 2	39 ± 1	4 7 ± 2	50± 1
	(22)	(22)	(22)	(15)	(13)	(10)

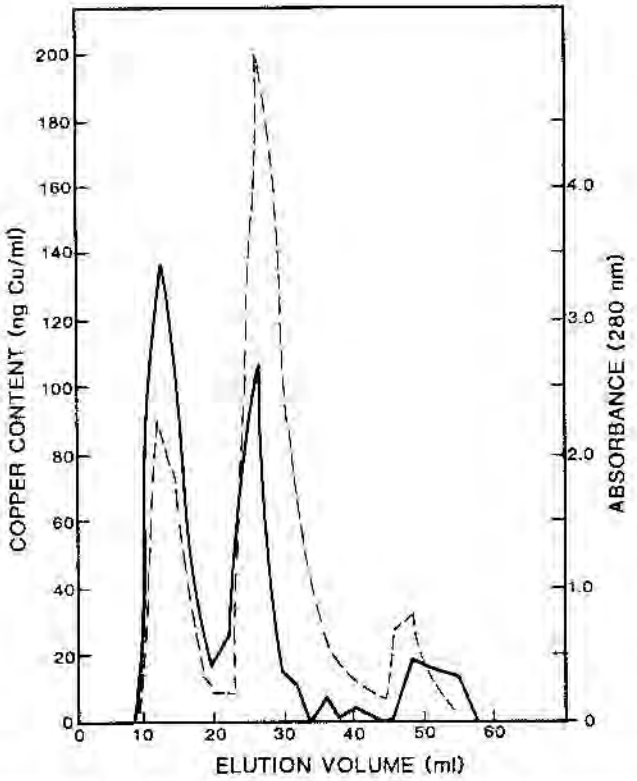
[a] *Numbers in parentheses* refer to small peaks; *dash* indicates no peak; *asterisk* indicates pronounced, very large peak.

Figure A6.2(12)-1



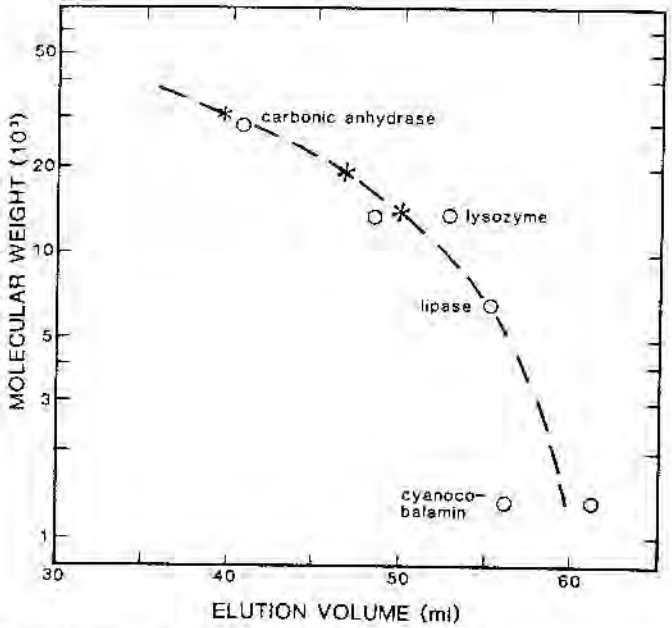
TEXT-FIGURE 1.—Fractionation of serum Cu components on columns of Sephadex G-150. Samples (1.0 ml) of fresh or previously frozen serum were applied to 50-ml gel columns, equilibrated with 0.9% NaCl. Fractions (0.9-1.1 ml) were collected and analyzed for Cu content by furnace atomic absorption spectroscopy (*see* "Materials and Methods"). Cu concentration at ppb (———). Absorbance at 280 nm (· · · ·) (*right vertical values*) and *p*-phenylenediamine oxidase activities [– – –, ceruloplasmin (CP) activity] of fractions are also indicated. Profile shown is for a normal adult male. *Arrows* indicate elution positions of transcuprein (TC), CP, and albumin (ALB).

Figure A6.2(12)-2



TEXT-FIGURE 3.—Fractionation of serum Cu components on affinity columns of Affi-gel blue. Samples (1.5–2.0 ml) of serum were diluted fivefold with 20 m*M* phosphate buffer (pH 7.0) and applied to columns equilibrated with the same buffer. Stepwise elutions were with the phosphate buffer containing 2 *M* NaCl (starting at 18 ml) and with the same buffer containing 0.5 *M* KSCN (starting at 42 ml). Fractions were analyzed for absorbance at 280 nm (– – – –) and for Cu (————) by furnace atomic absorption spectroscopy (*see* "Materials and Methods").

Figure A6.2(12)-3



TEXT-FIGURE 4.—Elution of low-mol-wt serum Cu components and standards on columns of Sephadex G-150. Log mol wt of carbonic anhydrase (28,140), lysozyme (13,370), lipase (6,670), and vitamin B12 (cyanocobalamin; 1,355) are plotted against elution volumes under the conditions used for serum samples (text-fig. 1). Elution of the three low-mol-wt serum Cu components is indicated by *asterisks*.

			Official use only
		67 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).* Campbell, C.H., Brown, R. and Linder, M.C. (1981). Circulating Ceruloplasmin is an Important Source of Copper for Normal and Malignant Animal Cells. Biochim. Biophys. Acta. **678**: 27-38 (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	
		68 GUIDELINES AND QUALITY ASSURANCE	
68.1	**Guideline study**	No. This was a non-regulatory study carried out to investigate the role of ceruloplasmin as a copper transport protein for malignant and normal cells. No guidelines are available to address this specific objective. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
68.2	**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)*	
68.3	**Deviations**	No. Not applicable to non-guideline studies (refer to section 4.3.6 for a general discussion of deviations and deficiencies). *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	X
		69 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.*	
69.1	**Test material**	Cu^{2+} as $^{64}Cu(NO_3)_2$ and $^{67}Cu(NO_3)_2$.	
69.1.1	Lot/Batch number	Not available	
69.1.2	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: 5.0/13	**A6.2(13), Distribution of copper**
69.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Not available
69.1.2.2 Purity	*Give purity in % of active substance* [redacted]
69.1.2.3 Stability	*Describe stability of test material* Not available
69.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* $^{64}Cu(NO_3)_2$ and $^{67}Cu(NO_3)_2$ (specific activities in the ranges 3 - 4 µCi/µg and 50 -200 µCi/µg, respectively).
69.2 Test Animals	*Non*-entry *field*
69.2.1 Species	Rat
69.2.2 Strain	Fischer
69.2.3 Source	Simonson Laboratories (Gilroy, CA).
69.2.4 Sex	Female
69.2.5 Age	3 – 4 months
69.2.6 Treatment of animals	Some test animals were made copper deficient by feeding them a 'low copper diet' for 4 weeks prior to being killed. Some rats were implanted subsutaneously with Dunning mammary tumour DMBA-5A, 1 – 3 weeks before being killed. For in-vivo studies of ceruloplasmin uptake, samples of radioactive ceruloplasmin, plasma or plasma fractions were administered intravenously and rats were killed 1 hour later by exsanguination. Blood was collected from the vena cava, and various organs removed, all for analysis of radioactivity and residual blood.
69.3 Procedures	Non-entry field
69.3.1 Cell cultures and procedures.	Most culture studies were performed with Ehrlich ascites tumour cells initially propagated in Swiss Webster mice. Culture flasks containing 30 ml 6% RPMI medium were inoculated with approximately 10^6 cells obtained from the mouse peritoneum, and cultures were grown in an atmosphere of 5% CO_2/95% air, at 37°C for 1–3 days prior to use. Cells were collected by scraping and centrifugation and resuspended in cold, serum-free RPMI, to yield a final concentration of 10^6 cells/ml. 1 ml aliquots in glass culture tubes were used for uptake studies involving radioactive ceruloplasmin and other plasma fractions, and incubated under the previously described conditions. At various times after the start of incubation, individual culture tubes were emptied onto glass fibre discs. Tubes were inoculated for another 5 minutes with 2.0 ml 0.25% trypsin in 0.001 mM EDTA, and rinsed 5 times with about 5 ml cold, 0.9% NaCl. Filters were placed in vials for radioactive counting. Primary cultures of muscle cells were prepared from adult Sprague Dawley rats, and grown in Dulbecco medium with 20% serum. Confluent cells were fused into myotubules after 10-14 days of culturing in petri dishes. For uptake studies, cells were preincubated for 1 hour in 2.0 ml RPMI medium with 10% horse serum, after which 50 µl

	radioactive ceruloplasmin or rat serum fraction were administered. After 1-2 hour incubations, individual petri dishes were washed with cold 0.9% NaCl, the cells scraped off and transferred to centrifuge tubes for further washing and counting. The radioactivity remaining in the medium and in initial washings was also counted.
69.3.2 Preparation of ceruloplasmin and control samples for intravenous injection.	For studies of copper uptake, rat plasma was collected from rats injected intraperitoneally 2-4 hours previously with 50-200 ìCi doses of either $^{64}Cu(NO_3)_2$ or $^{67}Cu(NO_3)_2$. To prepare ^{67}Cu-labelled ceruloplasmin, samples of plasma were treated with Chelex-100 ion exchange resin to remove the non-ceruloplasmin copper. Non-ceruloplasmin ^{67}Culabelled samples were prepared by adding 1-10 ìCi of radioactive $Cu(NO_3)_2$ to cold rat plasma. Alternatively, 1 ml portions of radioactive plasma were applied to a 50 ml column of Sephadex G-150, samples and columns having previously been equilibrated with 0.15 M NaCl, or 0.05 M acetate buffer, pH5.5, containing 0.5 M NaCl. 1 ml fractions were collected. Those containing the radioactive ceruloplasmin and radioactive albumin were pooled separately, sometimes concentrated by ultrafiltration, and 50 ìl aliquots administered to cultured cells. For preparation of ceruloplasmin labelled in the protein moiety, the protein was purified from rat plasma by DEAE-cellulose chromatography and gel filtration. After chromatography of equilibrated plasma on DEAE-cellulose and subsequent concentration by ultrafiltration, samples were separated on a 50 ml column of Sephadex G-150, re-equilibrated with 0.05 M acetate buffer, pH 5.5, and re-chromatographed on DEAE cellulose. Protein was determined by the Folin procedure, using bovine serum albumin as the standard. In polyacrylamide gel electrophoresis, using 5% acrylamide in the separating gel, a major band representing ceruloplasmin with a lower copper content (after ascorbate treatment) was visible with an R_F of migration of 0.60 relative to bromophenol blue. Based on gel scans, preparations were between 95 and 99% pure. For preparation of [^{3}H]leucine ceruloplasmin, the protein was purified from plasma of rats injected intraperitoneally with 100 ìCi L-[^{3}H]leucine (specific activity > 10 Ci/mmol) 15 to 20 minutes before killing. Alternatively, the protein moiety of ceruloplasmin was labelled with radioactive iodine by treating 1 mg pure ceruloplasmin with ^{125}I. [^{125}I]Ceruloplasmin was diluted with cold ceruloplasmin to a final specific activity of 2×10^6 cpm/mg.
69.3.3 Counting procedures for radioactive samples.	Tritium radioactivity in tissues was measured by scintillation counting and the contribution of residual blood subtracted based on determination of the haemoglobin content of tissue homogenates. ã-Emitting radioisotopes were measured directly by placing whole organs, or portions of organs, in vials for ã-counting using a ã Trac 1191 counter.
69.3.4 Fractionation of liver and heart cell organelles.	Livers or hearts from groups of 3-5 rats treated intravenously with radioactive Cu-labelled ceruloplasmin or plasma were pooled, homogenised, and fractionated by differential centrifugation. Radioactivity in the various cell fractions was counted and related quantitatively to the total cpm present in 1 g tissue at the start.

X

70 RESULTS AND DISCUSSION

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

70.1 Results

Non-entry field.

70.1.1 Uptake of Cu-labelled ceruloplasmin by normal and malignant tissues, in vivo.

In order to compare the tissue uptake of ceruloplasmin-bound and non-ceruloplasmin-bound copper, aliquots of plasma labelled *in vivo* with ^{64}Cu or ^{67}Cu and treated with Celex (ceruloplasmin copper), as well as aliquots of cold plasma to which radioactive $Cu(NO_3)_2$ was added (non-ceruloplasmin copper) were administered to rats intravenously. The results of copper uptake over 1 hour are shown in **Table A6.2(13)-1** for normal rats. Two parameters were calculated: (a) the relative concentration of radioactivity for the different tissues; and (b) the relative total uptake of copper per organ (specific activity x organ weight/total counts recovered in the tissues analysed).

Based on the relative cpm/g tissue, ceruloplasmin was a much better source of copper than the non-ceruloplasmin form for heart, spleen and brain. There was also a greater *total* uptake of radioactivity from ceruloplasmin over non-ceruloplasmin copper by heart, brain and spleen. The proportions entering the liver from the two sources appeared to be similar, whereas the kidney showed a preference for nonceruloplasmin copper. The clearance of both forms of copper from the blood appeared to be rapid, being almost complete after 1 hour.

The importance of ceruloplasmin as a source of copper is further emphasised by considering the ìg copper absorbed from the ceruloplasmin and non-ceruloplasmin samples. In the case of ceruloplasmin, 0.28 ìg Cu were administered and diluted in a total of 8.25 ìg Cu already present in the circulating ceruloplasmin pool. 82% of this copper was absorbed in 1 hour. This represents a total of 7.0 ìg Cu absorbed from this source, assuming equal labelling of all the copper atoms in ceruloplasmin. In contrast, only 0.03 ìg of labelled non-ceruloplasmin copper was given by adding $^{67}Cu(NO_3)_2$ tracer to cold plasma (in this case, almost all the radioactivity was associated with the non-ceruloplasmin fraction, **Figure A6.2(13)-1**). This 0.03 ìg Cu was diluted in 0.99 ìg non-ceruloplasmin copper (0.12 µg/ml x plasma volume), with 86% being removed from the blood within 1 hour. This represents a total of only 0.88 ìg Cu absorbed from this source.

Similar results were obtained with rats bearing large transplantable tumours (**Table A6.2(13)-2**). However, in this case the tumour absorbed a considerable portion of the radioactive copper administered in either form, leaving a much smaller percentage of the radioactive dose for uptake by other tissues.

The intracellular distribution of copper absorbed from both sources was investigated for liver and heart by differential centrifugation. The results of three separate studies, in which radioisotope copper was given intravenously to rats as ceruloplasmin or after its addition to cold plasma as $Cu(NO_3)_2$, are shown in **Table A6.2(13)-3**. From these data, it is apparent that the form of copper administered did not affect its gross intracellular distribution, or content within the mitochondrial fraction.

70.1.2 Uptake of ceruloplasmin labelled in the protein moiety.

To better understand the mechanism of ceruloplasmin-copper uptake, ceruloplasmin was labelled in the protein moiety *in vivo* with [^{3}H]leucine, or in vitro with ^{125}I. As shown in **Table A6.2(13)-4**, intravenous administration of pure radio-iodinated ceruloplasmin resulted in a substantial net uptake by liver, heart and kidney in normal rats. Over a period of 1 hour, 38% of the administered dose had

	disappeared from the plasma, implying a fairly rapid internalisation of the whole ceruloplasmin molecule, with the majority removed by liver and kidney. The rate of removal of this label was, however much slower than the rate of removal of radioactive copper from plasma preparations of ceruloplasmin (**Table A6.2(13)-1** and **Table A6.2(13)-2**). It amounted to an uptake of 0.95 mg ceruloplasmin protein (equivalent to 3.2 tg Cu based on 0.34% copper in ceruloplasmin), assuming full equilibration of the labelled material with the 2.19 mg ceruloplasmin protein present in normal rats (320 tg/ml x plasma volume). This is in contrast with the 7.0 tg Cu (equivalent to 2.1 mg ceruloplasmin protein) calculated to have been taken up by normal rats when labelled in the copper moiety (**Table A6.2(13)-1**). The lack of correspondence between the rates of uptake of ceruloplasmin copper and ceruloplasmin protein implies at least partial separation in the mechanisms of uptake. When radioiodinated ceruloplasmin was administered to copper-deficient rats, with and without implanted tumours (**Table A6.2(13)-5**), a more rapid loss of radioactivity from plasma was observed. However, this was largely accounted for by a diminished dilution of the label by endogenous ceruloplasmin, which was about one-third of normal. In this study, 0.94 amd 0.82 mg ceruloplasmin were absorbed by the rats with and without tumours, respectively; essentially the same amounts as in normal rats (**Table A6.2(13)-4**). To check whether the radioiodination had altered the pattern of tissue ceruloplasmin uptake, ceruloplasmin samples labelled by *in vivo* injection of [^{3}H]leucine were used. As shown **in Table A6.2(13)-6**, total uptake of ceruloplasmin protein was less rapid than in the case of ceruloplasmin-copper; 0.56 mg ceruloplasmin protein (equivalent to 1.9 tg Cu) were absorbed by these deficient rats over 1 hour.
70.1.3 Uptake of copper from ceruloplasmin by tumour cells in vitro.	Uptake of radioactive copper from ceruloplasmin and non-ceruloplasmin serum fractions was investigated using cells in tissue culture. The samples of ceruloplasmin and non-ceruloplasmin copper used were from plasma labelled in vivo and fractionated by gel filtration. **Figure A6.2(13)-2** shows the time-course of uptake of ^{67}Cu from about 5tg ceruloplasmin per 10^{6} Ehrlich ascites tumour cells, or from an equivalent amount of copper in the non-ceruloplasmin fraction. Tumour cells were observed to rapidly take up ceruloplasmin copper. They also took up copper from the non-ceruloplasmin fraction, but to a lesser extent. Experiments were also conducted with primary cultures of rat skeletal muscle. In this case, uptake of both forms of copper label was much less rapid, amounting to 0.7-1.8% of the ceruloplasmin copper over 1-2 hours, and 0.23-0.33% of the non-ceruloplasmin fraction. This corresponded to an uptake of 1.6-5.8 tg Cu from ceruloplasmin and 0.03-0.04 tg non-ceruloplasmin copper taken up per dish of cells. Ceruloplasmin was therefore confirmed as the best source of copper for these cells.
70.2 Discussion	The data presented here show that ceruloplasmin is a major source of copper for uptake by normal and malignant cells. Ceruloplasmin appears to be a more important copper donor than the nonceruloplasmin fraction of plasma for most tissues when it is considered that the two forms of tracer administered enter, and are diluted by, copper pools of disparate size; the ceruloplasmin pool being about 10

times as large as the other. Conclusions based only on % uptake of radioactivity can therefore give a false picture. A tentative summary of the actual amounts of copper (and ceruloplasmin protein) absorbed in the various studies is provided in table **A6.2(13)-7**. The values shown were calculated on the basis of reasonable assumptions. However, the exact amounts of Cu absorbed from the two plasma pools can only be regarded as approximate, as it is not possible to be certain that all of the copper atoms attached to the ceruloplasmin administered were equally labelled during the 2-4 hours allowed for in vivo incorporation. It is possible that a substantial proportion of the label was present on the molecule's 'chelexable site', and may perhaps have been even more loosely attached. When samples from rats injected with tracer ^{67}Cu two hours before death were checked, it was found that 46-49% of the label could be removed with Chelex. This indicates that at least half of the label was tightly bound and had been incorporated as part of the 6-7 essential copper atoms during ceruloplasmin synthesis in the liver. Consequently, the uptake of 82 - 96% of the radioactivity from ceruloplasmin (**Table A6.2(13)-1** and **Table A6.2(13)-2**) may represent a complete uptake of loosely-bound label plus a substantial uptake of copper bound more firmly to ceruloplasmin. At the very least, this should represent an uptake of well over 1 µg Cu over 1 hour, as compared with about 7 µg if equal labelling of all Cu atoms is assumed (**Table A6.2(13)-7**). Since in most of the studies, the plasma was pretreated with Chelex, uptake of labelled copper should have been of the firmly-bound variety, representing as much as 7 µg Cu/hour, and more in the case of tumour-bearing rats. These figures are in contrast with the maximum of 0.9 µg Cu/hour absorbed from the non-ceruloplasmin fraction, again assuming full equilibration of the copper atoms in the plasma pool. The preference for ceruloplasmin over albumin may be even greater than these considerations suggest, in that a portion of the radioactive non-ceruloplasmin copper administered may have been transformed by the liver into radioactive ceruloplasmin that was in turn absorbed by other tissues.

The data indicate that the relative avidity of different organs for the two forms of copper administered was about equal in the case of the liver in terms of radioactivity administered, but that there was a marked preference for ceruloplasmin in the case of heart, spleen and brain. Kidney, however, showed some preference for the non-ceruloplasmin form.

The fast-growing, undifferentiated mammary tumours bourne by the rats absorbed a substantial proportion of the radioactivity from both copper sources. On an absolute basis, however, ceruloplasmin probably contributed more, when the size of the two plasma copper pools is considered (**Table A6.2(13)-7**). A preference for ceruloplasmin copper was also evident when other, fast-growing tumour cells were examined in tissue culture (**Figure A6.2(13)-2**). Normal muscle cells in culture also preferred ceruloplasmin copper, but absorbed it at a lower rate.

Studies on the uptake of ceruloplasmin labelled in the protein moiety confirm that ceruloplasmin protein is absorbed by many tissues, but with particular avidity by liver and heart. The type of labelling used did not alter the results. Also, on the basis of µg ceruloplasmin protein absorbed, there was no acceleration of uptake during copper deficiency (**Table A6.2(13)-7**).

The uptake of ceruloplasmin protein was considerably less rapid than

uptake of ceruloplasmin-Cu. The exact relationship cannot be determined, due to the difficulty of not knowing the extent to which the tracer labelled all the Cu atoms equally. On the basis of protein-labelling, roughly 2 µg Cu were absorbed over 1 hour (**Table A6.2(13)-7**), while most likely 7 µg Cu were absorbed from radioactive copper labelled material (Chelex-treated plasma). This strongly suggests that the copper and protein moieties of ceruloplasmin are not absorbed in parallel, but rather that at least some of the copper on this molecule is removed and replaced more rapidly than the rest, and represents copper transported on ceruloplasmin for cellular uptake.

71 APPLICANT'S SUMMARY AND CONCLUSION

71.1 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 role of ceruloplasmin as a copper transport protein for normal and malignant cells. No guidelines are available to address this specific objective.

Animals, tumours and treatments: All whole-animal experiments were performed with 3 – 4 month old female, Fischer rats, some of which were made copper deficient by feeding them a 'low copper diet' for 4 weeks prior to killing. Some rats were implanted subcutaneously with Dunning mammary tumour DMBA-5A, 1-3 weeks before killing.

Cell cultures and procedures: Most culture studies were performed with Ehrlich ascites tumour cells propagated in Swiss Webster mice. Approx. 10^6 mouse peritoneal cells were cultured in 6% RPMI medium in a 5% CO_2/95% air atmosphere at 37°C for 1–3 days. Cells were collected, centrifuged and resuspended in serum-free RPMI at a final concentration of 10^6 cells/ml. 1 ml aliquots were used for uptake studies with radioactive ceruloplasmin and other plasma fractions, and incubated as described above. At various times after the start of incubation, individual culture tubes were emptied onto glass fibre discs. Tubes were inoculated for another 5 minutes with 2.0 ml 0.25% trypsin in 0.001 mM EDTA and rinsed 5 times with about 5 ml cold, 0.9% NaCl (rinses were emptied onto the filter). Filters were placed in vials for radioactive counting.

Muscle cell cultures were prepared from adult Sprague Dawley rats, and grown in Dulbecco medium with 20% serum. Confluent cells were fused into myotubules after 10-14 days. For uptake studies, cells were preincubated for 1 hour in 2.0 ml RPMI medium with 10% horse serum, after which 50 µl radioactive ceruloplasmin or rat serum fraction were administered. After 1-2 hours, petri dishes were washed with cold 0.9% NaCl, the cells scraped off and transferred to centrifuge tubes for washing and counting. Radioactivity remaining in the medium and in initial washings was also counted.

Preparation of ceruloplasmin and control samples for intravenous injection: For copper uptake studies, plasma was collected from rats injected i.p. 2-4 hours previously with 50-200 iCi of $^{64}Cu(NO_3)_2$ or $^{67}Cu(NO_3)_2$. To prepare ^{67}Cu-labelled ceruloplasmin, plasma samples were treated with Chelex-100 ion exchange resin to remove non-ceruloplasmin copper. Non-ceruloplasmin ^{67}Cu-labelled samples were prepared by adding 1-10 iCi of $^{67}Cu(NO_3)_2$ to cold plasma. Alternatively, 1 ml portions of radioactive plasma were applied to 50 ml Sephadex G-150 columns; samples and columns having previously been

		equilibrated with 0.15 M NaCl, or 0.05 M acetate buffer, pH5.5, containing 0.5 M NaCl. 1 ml fractions were collected. Those containing radioactive ceruloplasmin and radioactive albumin were pooled separately, concentrated by ultrafiltration, and 50 ìl aliquots administered to cultured cells. For preparation of ceruloplasmin labelled in the protein moiety, protein was purified from rat plasma by DEAE-cellulose chromatography and gel filtration. After chromatography of equilibrated plasma on DEAE-cellulose and concentration by ultrafiltration, samples were separated on a 50 ml Sephadex G-150 column, re-equilibrated with 0.05 M acetate buffer, pH 5.5, and re-chromatographed on DEAE cellulose. Protein was determined by the Folin procedure, using bovine serum albumin as standard. In polyacrylamide gel electrophoresis, using 5% acrylamide in the separating gel, a major band representing ceruloplasmin with a lower copper content (after ascorbate treatment) was visible with an R_F of migration of 0.60 relative to bromophenol blue. Based on gel scans, preparations were between 95 and 99% pure. For preparation of [^{3}H]leucine ceruloplasmin, protein was purified from plasma of rats injected i.p. with 100 ìCi L-[^{3}H]leucine (specific activity > 10 Ci/mmol) 15-20 minutes before killing. Alternatively, the protein moiety was labelled with radioactive iodine by treating 1 mg pure ceruloplasmin with ^{125}I. [^{125}I]Ceruloplasmin was diluted with cold ceruloplasmin to a final specific activity of 2 x 10^6 cpm/mg. ***Counting procedures for radioactive samples:*** Tritium radioactivity in tissues was measured by scintillation counting and the contribution of residual blood subtracted on the basis of haemoglobin content of tissue homogenates. ã-Emitting radioisotopes were measured directly by placing whole organs, or portions of organs, in vials for ã-counting. ***Fractionation of liver and heart cell organelles:*** Livers or hearts from groups of 3-5 rats treated i.v. with radioactive Cu-labelled ceruloplasmin or plasma were pooled, homogenised, and fractionated by differential centrifugation. Radioactivity in the cell fractions was counted and related to the total cpm present in 1 g tissue.	X
71.2	**Results and discussion**	*Summarize relevant results; discuss dose-response relationship.* ***Uptake of Cu-labelled ceruloplasmin by normal and malignant tissues, in vivo:*** In order to compare tissue uptake of ceruloplasminbound and non-ceruloplasmin-bound copper, aliquots of plasma labelled *in vivo* with ^{64}Cu or ^{67}Cu and treated with Celex (ceruloplasmin copper), or aliquots of cold plasma to which radioactive $Cu(NO_3)_2$ had been added (non-ceruloplasmin copper), were administered to rats i.v. It was found that, based on the relative cpm/g tissue, ceruloplasmin was a better source of copper than the non-ceruloplasmin form for heart, spleen and brain. There was also a greater *total* uptake of radioactivity from ceruloplasmin over non-ceruloplasmin copper by these organs. The proportions entering the liver from the two sources appeared to be similar, whereas kidney showed a preference for non-ceruloplasmin copper. Clearance of both forms of copper from the blood appeared to be rapid, being almost complete after 1 hour. The importance of ceruloplasmin as a source of copper was emphasised by considering the ìg copper absorbed from ceruloplasmin and non-ceruloplasmin samples. In the case of ceruloplasmin, 0.28 ìg Cu were administered and diluted in a total of 8.25 ìg Cu already present in the circulating ceruloplasmin pool. 82% of this copper was absorbed in 1	

hour, representing a total of 7.0 tg Cu absorbed (assuming equal labelling of all ceruloplasmin copper atoms). In contrast, only 0.03 tg of labelled non-ceruloplasmin copper was given by adding $^{67}Cu(NO_3)_2$ tracer to cold plasma. In this case, nearly all the radioactivity became associated with the non-ceruloplasmin fraction. This 0.03 tg Cu was diluted in 0.99 tg non-ceruloplasmin copper (0.12 tg/ml x plasma volume), with 86% removed from the blood within 1 hour. This represents a total of only 0.88 tg Cu absorbed.

Similar results were obtained with rats bearing transplantable tumours. However, in this case the tumour absorbed a considerable portion of the radioactive copper administered in either form, leaving a smaller % of the dose for uptake by other tissues.

Intracellular distribution of copper absorbed from both sources was investigated for liver and heart by differential centrifugation. From the results of 3 separate studies, in which radioisotope copper was given i.v. to rats as ceruloplasmin or after its addition to cold plasma as $Cu(NO_3)_2$, it is apparent that the form of copper administered did not affect gross intracellular distribution, or content within the mitochondrial fraction.

Uptake of ceruloplasmin labelled in the protein moiety:
Ceruloplasmin was labelled in the protein moiety *in vivo* with [^{3}H]leucine, or in vitro with ^{125}I. Intravenous administration of radio-iodinated ceruloplasmin resulted in a substantial net uptake by liver, heart and kidney in normal rats. Over a period of 1 hour, 38% of the dose had disappeared from plasma, implying fairly rapid internalisation of the whole ceruloplasmin molecule, with the majority removed by liver and kidney. The rate of removal of this label was, however, much slower than the rate of removal of radioactive copper from plasma preparations of ceruloplasmin. This amounted to an uptake of 0.95 mg ceruloplasmin protein (equivalent to 3.2 tg Cu based on 0.34% copper in ceruloplasmin), assuming full equilibration of the labelled material with the 2.19 mg ceruloplasmin protein present in normal rats (320 tg/ml x plasma volume). This is in contrast with the 7.0 tg Cu (equivalent to 2.1 mg ceruloplasmin protein) calculated to have been taken up by normal rats when labelled in the copper moiety. The lack of correspondence between the rates of uptake of ceruloplasmin copper and protein implies partial separation in the mechanisms of uptake.

When radioiodinated ceruloplasmin was administered to copperdeficient rats, with and without implanted tumours, a more rapid loss of radioactivity from plasma was observed. However, this was largely accounted for by a diminished dilution of the label by endogenous ceruloplasmin, which was about one-third of normal. In this study, 0.94 and 0.82 mg ceruloplasmin were absorbed by the rats with and without tumours, respectively; essentially the same amounts as in normal rats.

To check whether radioiodination altered the pattern of tissue ceruloplasmin uptake, samples labelled by *in vivo* injection of [^{3}H]leucine were used. Uptake of ceruloplasmin protein was less rapid than for ceruloplasmin-copper, with 0.56 mg ceruloplasmin protein (equivalent to 1.9 tg Cu) being absorbed by deficient rats over 1 hour.

Uptake of copper from ceruloplasmin by tumour cells in vitro: The samples of ceruloplasmin and non-ceruloplasmin copper used were from plasma labelled *in-vivo* and fractionated by gel filtration. Tumour cells were observed to rapidly take up ceruloplasmin copper. They also took up copper from the non-ceruloplasmin fraction, but to a lesser extent.

In experiments with primary cultures of rat skeletal muscle, uptake of both forms of copper label was much less rapid, amounting to 0.7-1.8% of the ceruloplasmin copper over 1-2 hours, and 0.23-0.33% of the non-ceruloplasmin fraction. This corresponded to an uptake of 1.6-5.8 tg Cu from ceruloplasmin and 0.03-0.04 tg non-ceruloplasmin copper taken up per dish of cells. Ceruloplasmin was therefore confirmed as the best source of copper for these cells.

Discussion: Ceruloplasmin is a major source of copper for uptake by normal and malignant cells. The data indicate that the relative avidity of different organs for the two forms of copper was about equal in the case of the liver in terms of radioactivity administered, but that there was a marked preference for ceruloplasmin in the case of heart, spleen and brain. Kidney, showed some preference for non-ceruloplasmin copper.

Fast-growing, undifferentiated mammary tumours absorbed a substantial proportion of the radioactivity from both copper sources. On an absolute basis, however, ceruloplasmin probably contributed more. A preference for ceruloplasmin copper was also evident when other, fast-growing tumour cells were examined in tissue culture. Normal muscle cells in culture also preferred ceruloplasmin copper, but absorbed it at a lower rate.

Studies on the uptake of ceruloplasmin labelled in the protein moiety confirm that ceruloplasmin protein is absorbed by many tissues, but with particular avidity by liver and heart. The type of labelling used did not alter the results. Also, on the basis of tg ceruloplasmin protein absorbed, there was no acceleration of uptake during copper deficiency.

Uptake of ceruloplasmin protein was considerably less rapid than that of ceruloplasmin-Cu. This suggests that the copper and protein moieties of ceruloplasmin are not absorbed in parallel, but that some of the copper on this molecule is removed and replaced more rapidly than the rest, representing copper transported on ceruloplasmin for cellular uptake.

71.3 Conclusion

Cells in vivo and in vitro will take up copper to varying extents from whatever source copper is offered, whether it is bound to ceruloplasmin or other plasma components (ionic). Ceruloplasmin has, however, been confirmed as the major source of copper for most tissue types.

71.3.1 Reliability

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

2

71.3.2 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.

(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: 5.0/13	**A6.2(13), Distribution 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	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	[redacted]

Table A6.2(13)-1

Tissue Uptake of Copper from Ceruloplasmin and Non-Ceruloplasmin Plasma Fractions after Intravenous Adminstration to Normal Rats.

Rats were injected by tail vein with ^{67}Cu labelled ceruloplasmin in plasma (free copper removed by Chelex) or cold plasma which tracer ^{67} Cu $(NO_3)_2$ had been added, 1 h before death. The contribution of ^{67}Cu in trapped blood in the tissues has been subtracted. Values are given as mean ± S. D., four and three rats in the **two** groups, respectively. Body weight of rats was 188 ± 8g. The total plasma Cu in such animals is 1.62 tg/ml as determined previously by anodic stripping voltammetry. In this study, the liver absorbed 33 and 47% of the dose administered via the two sources, respectively.

Table A6.2(13)-2

Tissue Uptake of Copper from Ceruloplasmin and other Fractions of Plasma after Intravenous Administration to rats Bearing Subcutaneous Mammary Tumor DMBA-5A

Groups of two rats similar to those used in the previous study (Table A6.2.**13**-1) were used. All parameters were as in Table A6.2.**13**-1 except that (a) rats bore large, subcutaneous tumours, and (b) ^{64}Cu was the radionuclide. For nonceruloplasmin, 1.3µg copper were administered, per rat, as compared with about 1.1µg Cu as ceruloplasmin. 100-fold less radioactivity were administered in the case of ceruloplasmin vs. non-ceruloplasmin copper. Body weight of rats was 173 ±18 g and total plasma Cu of comparable animals 2.52µg/ml (10). In this experiment, the liver took up 26% of the radioactivity from both copper sources.

Table A6.2(13)-4

Tissue Uptake of ^{125}I-Ceruloplasmin After its Intravenous Administration to Normal Rats

Samples (0.3mg) of pure, radioiodinated, rat ceruloplasmin were administered to four normal rats by tail vein injection, 1 h before death. Data were calculated and are presented as in Table A6.2.**13**-1. Rats weighed 171 ± 5 g. In this study, the liver adsorbed 9.0% of the injected dose. No significant brain uptake occurred.

Table A6.2(13)-5

Tissue Uptake of Ceruloplasmin, labelled in Vitro with ^{125}I, after Intravenous Administration to Copper-Deficient Rats With and Without Transplantable Tumours.

Pure samples (0.6 mg/rat) of radioiodinated rat ceruloplasmin were administered to copper deficient rats, with (2 rats), and without (3 rats), large subcutaneous mammary tumours (DMBA-5A), 1 h before death. Data are treated and presented as described in Table A6.2.**13**-1. In these experiments, no significant brain uptake occurred. Rats weighed 210 ± 15 g, including tumours. Total plasma Cu was not determined; ceruloplasmin oxidase activity was 1/3 of normal in the controls. In this study, the liver absorbed 2.2 and 3.1% of the injected dose, for the two groups, respectively.

Table A6.2(13)-6

Tissue Uptake of Ceruloplasmin Labelled in Vivo with (^{3}H) Leucine, after Intravenous Administration to Copper-Deficient Rats with and Without Transplantable Tumours

Pure samples (0.10 mg/rat) of ceruloplasmin, isolated from rats pre-injected with (^{3}H) leucine, were administered intravenously by tail vein to groups of four rats, with and without small, transplanted mammary tumours (DMBA-5A), 1 h before death. Data are treated and presented as in Table A6.2.**13**-1. Rats weighed 183 ± 18g, including tumours. In these studies, liver absorbed 25 and 17% of the injected dose in rats with and without tumours, respectively.

COMMENTS FROM ...

Date	*Give date of comments submitted*

Table A6.2(13)-1

Tissue Uptake of Copper from Ceruloplasmin and Non-Ceruloplasmin Plasma Fractions after Intravenous Adminstration to Normal Rats.

Rats were injected by tail vein with ^{67}Cu labelled ceruloplasmin in plasma (free copper removed by Chelex) or cold plasma which tracer $^{67}Cu(NO_3)_2$ had been added, 1 h before death. The contribution of ^{67}Cu in trapped blood in the tissues has been subtracted. Values are given as mean ± S. D., four and three rats in the groups, respectively. Body weight of rats was 188 ± 8g. The total plasma Cu in such animals is 1.62 tg/ml as determined previously by anodic stripping voltammetry. In this study, the liver absorbed 33 and 47% of the dose administered via the two sources, respectively.

Copper source:	Tissue ^{67}Cu concentration (% of dose/g)		Percent of total uptake [b]		Organ weight [a] (g or ml)
	Ceruloplasmin	Non-ceruloplasmin	Ceruloplasmin	Non-ceruloplasmin	
Tissue:					
Liver	6.6 ± 1.0	9.4 ± 0.9	74 ± 10	64 ± 1	5.0 ± 0.2 (7)
Heart	5.6 ± 1.0	2.9 ± 0.4	6 ± 1	2 ± 0	0.44 ± 0.02
Kidney	4.1 ± 5.6	12.1 ± 3.9	10 ± 10	27 ± 3	1.3 ± 0.1
Spleen	8.6 ± 3.6	4.9 ± 0.8	9 ± 4	5 ± 3	0.42 ± 0.04
Brain	0.5 ± 0.1	0.3, 0.4 (2)	2 ± 0	0.9, 0.9	1.7 ± 0.2
Plasma	2.4 ± 1.1	1.9 ± 0.3	(18 ± 4)[c]	(14 ± 10)[c]	7.5 ± 0.3 [d]

[a] Mean cpm/organ-organ weight (in g).
[b] Total uptake for a given tissue relative to the total in the tissues shown (not including plasma).
[c] Percent of total injected dose remaining in the plasma.
[d] Based on 4% of body weight.

Table A6.2(13)-2

Tissue Uptake of Copper from Ceruloplasmin and other Fractions of Plasma after Intravenous Administration to rats Bearing Subcutaneous Mammary Tumor DMBA-5A

Groups of two rats similar to those used in the previous study (Table A6.2.9-1) were used. All parameters were as in Table A6.2.9-1 except that (a) rats bore large, subcutaneous tumours, and (b) ^{64}Cu was the radionuclide. For nonceruloplasmin, 1.3µg copper were administered, per rat, as compared with about 1.1µg Cu as ceruloplasmin. 100-fold less radioactivity were administered in the case of ceruloplasmin vs. non-ceruloplasmin copper. Body weight of rats was 173 ±18 g and total plasma Cu of comparable animals 2.52µg/ml (10). In this experiment, the liver took up 26% of the radioactivity from both copper sources.

Copper Source:	Tissue 64 Cu concentration (% of dose/g)		Percent of total uptake [b]		Organ [a]
	Ceruloplasmin	Non-ceruloplasmin	Ceruloplasmin	Non-ceruloplasmin	
Tissue:					
Tumor	1.0, 1.3	1.2, 0.9	60, 43	54, 54	37 ± 10 (7)
Liver	3.9, 5.6	5.4, 4.1	31, 45	37, 28	5.5 ± 0.1
Heart	4.0, 5.8	0.3, 0.2	3,4	0.2, 0.1	0.5 ± 0.1
Kidney	2.9, 4.2	5.0, 4.1	5,8	0.7	1.3 ± 0.1
Brain	0.9, 1.4	0.05, 0.04	1,1	0,0	1.3 ± 0.1
Plasma	0.6, 0.4	1.4, 1.4	(4, 3) [c]	(10, 10)[c]	6.9 ± 0.7 [d]

a Mean cpm/organ-organ weight (in g).
b Total uptake for a given tissue relative to the total in the tissues shown (not including plasma).
c Percent of total injected dose remaining in the plasma.
d Based on 4% of body weight.

Table A6.2(13)-3

Intracellular Distribution of Radioactive copper after its intravenous administration as Ceruloplasmin or ionic copper added to plasma.

Samples of ^{67}Cu or ^{64}Cu-ceruloplasmin labelled by in vivo incorporation of radioactivity, or of cold plasma to which radioactive copper ($Cu(NO_3)_2$) was added, in vitro, were administered to normal rats by tail vein injection 1 hr before death. Tissues from groups of 2-4 rats were pooled, homogenized in 0.25 M sucrose, and subjected to differential contrifugation. The results are from three separate experiments (mean ± S.D.).

Form of radiocopper administered:	Distribution of ^{67}CU (% tissue cpm)	
	Ceruloplasmin	Plasma + ionic copper
Tissue:		
Liver		
Nuclear pellet	39 ± 6	42 ±7
Mitochondrial fraction	9 ± 5	10 ±5
Post-mitochondrial supernatant	50 ± 7	49 ±3
Heart		
Nuclear pellet	46 ± 9	38 ±5
Mitochondrial fraction	6 ± 2	6 ±2
Post-mitochondrial supernatant	46 ± 15	55 ±6

Table A6.2(13)-4

Tissue Uptake of ^{125}I-Ceruloplasmin After its Intravenous Administration to Normal Rats

Samples (0.3mg) of pure, radioiodinated, rat ceruloplasmin were administered to four normal rats by tail vein injection, 1 h before death. Data were calculated and are presented as in Table A6.2.9-1. Rats weighed 171 ± 5 g. In this study, the liver adsorbed 9.0% of the injected dose. No significant brain uptake occurred.

Tissue:	Tissue concentration of radio-activity (% of dose/g)	Percent total uptake [b]	Organ weight (g or ml)
Liver	1.5 ± 0.2	65± 3	5.9± 0.4
Heart	0.9 ± 0.1	3± 1	0.5± 0.0
Kidney	2.9 ± 0.3	28± 2	1.4± 0.0
Spleen	1.5 ± 0.1	4± 1	0.4± 0.0
Plasma	9.3 ± 2.5	(62± 3)[c]	(6.7 ± 0.4)[d]

a Mean cpm/organ-organ weight (in g).
b Total uptake for a given tissue relative to the total in the tissues shown (not including plasma).
c Percent of total injected dose remaining in the plasma.
d Based on 4% of body weight.

Table A6.2(13)-5

Tissue Uptake of Ceruloplasmin, labelled in Vitro with ^{125}I, after Intravenous Administration to Copper-Deficient Rats With and Without Transplantable Tumours.

Pure samples (0.6 mg/rat) of radioiodinated rat ceruloplasmin were administered to copper deficient rats, with (2 rats), and without (3 rats), large subcutaneous mammary tumours (DMBA-5A), 1 h before death. Data are treated and presented as described in Table A6.2.9-1. In these experiments, no significant brain uptake occurred. Rats weighed 210 ± 15 g, including tumours. Total plasma Cu was not determined; ceruloplasmin oxidase activity was 1/3 of normal in the controls. In this study, the liver absorbed 2.2 and 3.1% of the injected dose, for the two groups, respectively.

Type of rat:	Tissue concentration of radioactivity (% of dose/g)		Percent of total uptake[b]		Organ weight (g or ml)
Tissue:	Normal	+Tumour	Normal	+Tumour	
Tumour	-	0.32, 0.35	-	63, 74	26, 28 (2)
Liver	0.33 ± 0.00	0.47, 0.46	64 ± 2	22, 23	6.7 ± 0.4 (5)
Heart	0.35 ± 0.12	0.00, 0.00	7 ± 2	0,0	0.5 ± 0.0
Kidney	0.59 ± 0.13	0.85, 0.74	26 ± 2	7, 8	1.3 ± 0.1 [e]
Spleen	0.22 ± 0.07	0.24, 0.11	3 ± 1	2, 1	0.8 ± 0.4
Plasma	3.33 ± 0.24	3.69, 4.04	(28 ± 1)[c]	(31,34)[c]	(8.4 ± 0.6)

a Mean cpm/organ-organ weight (in g).
b Total uptake for a given tissue relative to the total in the tissues shown (not including plasma).
c Percent of total injected dose remaining in the plasma.
d Based on 4% of body weight.
e Kidney weight was significantly reduced, and spleen weight significantly increased, in the tumour-bearing rats.

Table A6.2(13)-6

Tissue Uptake of Ceruloplasmin Labelled in Vivo with (^{3}H) Leucine, after Intravenous Administration to Copper-Deficient Rats with and Without Transplantable Tumours

Pure samples (0.10 mg/rat) of ceruloplasmin, isolated from rats pre-injected with (^{3}H) leucine, were administered intravenously by tail vein to groups of four rats, with and without small, transplanted mammary tumours (DMBA-5A), 1 h before death. Data are treated and presented as in Table A6.2.9-1. Rats weighed 183 ± 18g, including tumours. In these studies, liver absorbed 25 and 17% of the injected dose in rats with and without tumours, respectively.

Type of rat:	Tissue concentration of radioactivity (% of dose/g)		Percent of total uptake[b]		Organ weight (g or ml)
	Controls	+Tumour	Control	+ Tumour	
Tissue:					
Tumour	-	2.0 ± 1.0	-	9 ± 4	1.1 ± 0.4 (4)
Liver	4.5 ± 0.6	3.0 ± 0.2	58 ± 1	66 ± 7	5.6 ± 1.0 (8)
Heart	7.1 ± 1.6	2.5 ± 1.7	10 ± 1	5 ± 3	0.53 ± 0.06
Kidney	5.8 ± 1.3	1.6 ± 0.2	18 ± 1	8 ± 3	1.2 ± 0.2
Spleen	9.4 ± 4.0	0.9 ± 0.2	8 ± 4	1 ± 1	0.34 ± 0.06
Brain	2.0 ± 0.9	1.2 ± 0.3	7 ± 3	9 ± 3	1.6 ± 0.2
Plasma	3.0 ± 1.2	4.7 ± 0.5	(22 ± 7) [c]	(34 ± 3) [c]	7.3 ± 0.7 [d]

a Mean cpm/organ-organ weight (in g).
b Total uptake for a given tissue relative to the total in the tissues shown (not including plasma).
c Percent of total injected dose remaining in the plasma.
d Based on 4% of body weight.

Table A6.2(13)-7

Summary: Calculated Total Uptake of Copper (and ceruloplasmin protein) in the Vivo Studies.

Mean total uptake of ceruloplasmin copper (and protein), and of non-ceruloplasmin copper was calculated based on the size of these copper pools, the doses administered and the percentage of the dose remaining in the plasma 1h after its administration, as described in the text to the Results. Assumptions used were that (a) ceruloplasmin copper is 90% of the normal plasma pool; (b) the concentration of ceruloplasmin protein is 32 mg/dl in normal rats and one-third that value in deficient ones-based on assays of enzyme activity; (c) ceruloplasmin contains 0.34% copper, by weight; and (d) in tumour-bearing rats the non-ceruloplasmin copper pool is still 10% of the total. Also, the data shown assume there was equal labelling of all copper atoms in each of the plasma copper pools used to trace uptake.

		Total uptake	
Plasma fraction administered: Reference:	**Non-ceruloplasmin (tag Cu)**	**Ceruloplasmin (tag Cu)**	**(mg protein)**
Table I normal rats	0.86	7.0	(2.1) [a]
Table II tumour-bearing rats	1.69	15	(4.4)
Table IV normal rats	-	(3.2) [b]	0.95
Tables V, VI deficient rats	-	(3.1, 1.9)	0.91, 0.56

[a] Calculated from the μg Cu absorbed, assuming ceruloplasmin contains 0.34% Cu.
[b] Calculated from the mg ceruloplasmin protein absorbed.

Figure A6.2(13)-1

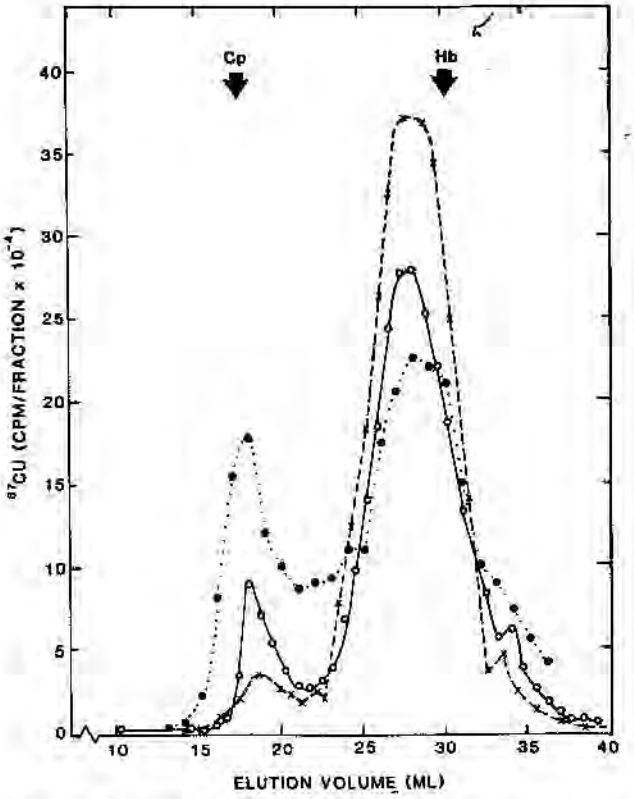


Fig. 2. Separation of ^{67}Cu-labeled ceruloplasmin and albumin by gel filtration. Samples of plasma (1.0 ml), from rats injected intraperitoneally with $^{67}Cu(NO_3)_2$ 2 h before death (●·····●), from rats intubated in the stomach with $^{67}Cu(NO_3)_2$ 10 min before death (o——o), or 'cold' plasma to which 10 μl of $^{67}Cu(NO_3)_2$ had been added directly (X-----X), were applied to 50 ml columns of Sephadex G-150 equilibrated with 0.9% NaCl . 1-ml fractions collected were monitored for radioactivity. Radioactivity per fraction is plotted against elution volume (ml). The elution volumes for pure rat ceruloplasmin (Cp) and bovine hemoglobin (Hb) on the same column are indicated.

Figure A6.2(13)-2

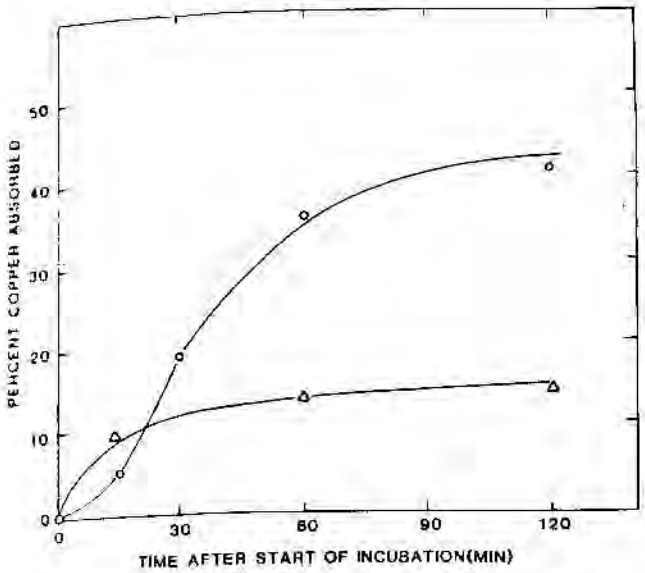


Fig. 3. Uptake of ^{67}Cu from ceruloplasmin and non-ceruloplasmin fractions of plasma by tumor cells in tissue culture. Sample (50 μl) of pooled material of the type obtained in Fig. 2 (●·····●) were added to 10^6 Ehrlich ascites tumor cells in 1.0 ml of nonserum-containing RPMI medium. Radioactivity retained by the washed cells at various times after the start of incubation was monitored. o——o, ceruloplasmin-copper; Δ——Δ, non-ceruloplasmin-copper.

			Official use only
		72 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 den Berg, G.J., Van Wouwe, J.P and Beynen, A.C., (1990). Ascorbic Acid Supplementation and Copper Status in Rats. Biological Trace Element Research, **23**: 165-172 (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	
		73 GUIDELINES AND QUALITY ASSURANCE	
73.1	**Guideline study**	No. This was a non-regulatory study carried out in rats to investigate the effects of high ascorbic acid loads on Cu concentrations in various tissues and on Cu^{64} retention in rats. The effects of the high ascorbic acid diets were compared with those of a low Cu diet. The study consisted of two separate experiments. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
73.2	**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)*	
73.3	**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")*	
		74 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.	
74.1	**Test material**	Cu^{2+} as copper sulphate. ^{64}Cu in sodium acetate buffer.	X
74.1.1	Lot/Batch number	Not available	

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/14	**A6.2(14), Homeostasis of copper**

74.1.2 Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*
74.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* See section 3.1
74.1.2.2 Purity	*Give purity in % of active substance* [redacted]
74.1.2.3 Stability	*Describe stability of test material* Not available
74.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* ^{64}Cu (specific activity 20.2 TBq/mol, HOR, Interfacility Reactor Institute, Delft, The Netherlands. Non-entry field
74.2 Test Animals	
74.2.1 Species	Rats
74.2.2 Strain	Wistar rats of the Hsd/Cpb strain.
74.2.3 Source	Harlan-CPB, Zeist, the Netherlands.
74.2.4 Sex	Male
74.2.5 Age/weight/height at study initiation	*Young adults recommended* *Experiment 1:* Age not stated; bodyweight 200 g. *Experiment 2:* Age not stated; bodyweight 80 g. 74.2.6 Number of animals *Give number*
per group	*Specify, if there are differences for example for treatment and recovery groups* *Experiment 1:* 5 animals per group. Experiment 2: 4 or 8 animals per group.
74.2.7 Controls	Yes
74.3 Administration/ Exposure	*(fill in respective route in the following, delete other routes)* *Experiment 1 :* ^{64}Cu was administered orally in the diet.
	Experiment 2: Copper sulphate was administered orally in the diet ; ^{64}Cu was administered intraperitoneally (i.p.). X
74.3.1 Duration of treatment	*Experiment 1:* Test animals received a single oral dose of ^{64}Cu. Faecal output was monitored for 3 days.
	Experiment 2: 45 days. X
74.3.2 Exposure scenario	Test animals were individually housed in cages in a room with controlled temperature (19-21°C), relative humidity (50-60%) and lighting (light 06:00–18:00). Food and water were provided *ad libitum*. *Experiment 1*: The recovery of orally administered radioactive Cu from faeces was determined with a dose of ^{64}Cu. Each rat was given by stomach tube 0.8 t mol/kg of ^{64}Cu in a total volume of 0.25 ml 50 mM sodium acetate buffer, pH 5.6, with or without 140 tmol ascorbic acid. Faeces of each rat was collected quantitatively for the measurement of

	^{64}Cu activity. *Experiment 2:* On day 0 of this experiment, the animals were randomly divided into 3 groups consisting of 4 to 8 animals each. Two groups were fed a Cu-adequate diet (containing 150 jtmol/kg) with or without 56.8 mmol/kg (w/w) ascorbic acid. A third group served as a positive control and was fed a Cu-deficient diet (containing 5 jtmol/kg). Food and water were supplied *ad libitum*. On day 35, each rat was injected intraperitoneally with a dose of ^{64}Cu. Whole body counting was performed by placing a container with the animal into a tank filled with a scintillation liquid, equipped with a photomultiplier connected to a multichannel analyser. The efficiency of this counter for ^{64}Cu was 14%. Whole body counting was performed 2 hours post-injection and the daily for 4 days. The measured wholebody ^{64}Cu activities at any time, R(t), and those measured 2 hours post-injection, R_0, were used to calculate the ln% of the administered dose ln% dose = ln{R(t)/R0x100}. The ln% dose was plotted vs time, t, and a linear relation from day 2 – 4 was found. By a least square fit, the slope (X) and the intercept, the % apparent retention (R_η), were computed. The biological half-life (T_b) was calculated as $T_b = \ln 2/X$. On day 41, rats were again injected i.p. with ^{64}Cu . The next day, blood was collected and the animals were sacrificed. Plasma was collected by low-speed centrifugation. Liver, left tibia, and left flexor digitorium longus muscle were removed for determination of radioactivity and Cu concentration. Whole-body contents of Cu were assessed using values of analysed Cu in liver, plasma, muscle, and bone and values for the mass of these tissues, whereas Cu in locations such as gastrointestinal tract, brain and fur were not accounted for. The specific activity was calculated from ^{64}Cu activity in Bq divided by Cu content in mmol.	X
74.4 Examinations	Non-entry field	
74.4.1 Body weight	*yes/no (give time periods for determinations).* Yes. Bodyweight was monitored on days 0 and 42 of Experiment 2.	
74.4.2 Blood collections	*yes/no (give time periods for determinations).* Yes. Blood was collected for analysis of ceruloplasmin on day 42 of Experiment 2.	
74.4.3 Faeces collections	*yes/no (give time periods for determinations).* Faeces was collected quantitatively for analysis of Cu over the course of Experiment 1.	
74.4.4 Tissues	*yes/no (give time periods for determinations).* Yes. Tissue samples were collected for analysis of Cu on day 42 of Experiment 2.	
3.5 Sample processing and analysis	Non-entry field.	
3.5.1 Copper analysis	^{64}Cu activity in tissues was measured in a gamma counter. Corrections were made for decay and background. Total Cu in diets and tissues were determined in duplicate (after wet digestion with 1.0 ml of 65% nitric acid and 0.5 ml of 30% hydrogen peroxide) by atomic absorption spectrometry.	
3.5.2 Ceruloplasmin analysis	Ceruloplasmin in plasma was assayed as *p*-phenylenediamine oxidase activity.	
3.5.3 Statistical analysis	Results of Cu analyses were given as mean ± S.D. Statistical analyses were performed by the two-tailed Student's t-test and 0.05 was considered the maximum value for the type 1 error.	