	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.
55.3.2 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.
55.3.3 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.
55.3.4 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.
55.3.5 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 m1 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.

55.3.6 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 serurn 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.
	56 RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are given below.*
56.1 Results	Non-entry field
56.1.1 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.	
56.1.2 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
56.1.3 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.

56.2 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 $^{67}Cu^{2+}$ can bind for detection.

57 APPLICANT'S SUMMARY AND CONCLUSION

57.1 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**

57.2 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.

57.3 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.

57.3.1 Reliability

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

2

57.3.2 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	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

	COMMENTS FROM ...
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

58 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

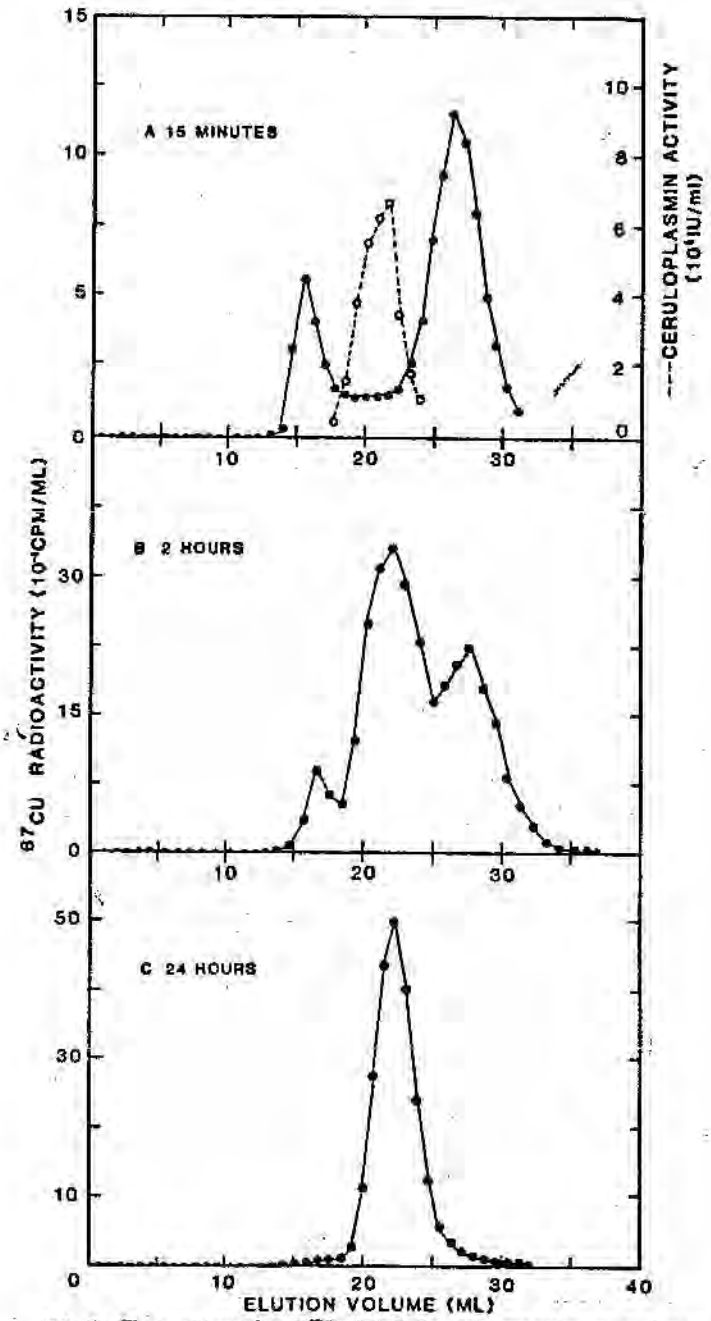


FIG. 1. Chromatography of ^{67}Cu-labeled rat plasma taken at various times after radioisotope administration. Pairs of rats were injected with 250–400 μCi of $^{67}CuCl_2$ in 0.9% NaCl and killed after 15 min (*A*) and 2 (*B*) and 24 h (*C*). Pooled samples (1.0 ml) of heparinized plasma from 2 rats (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

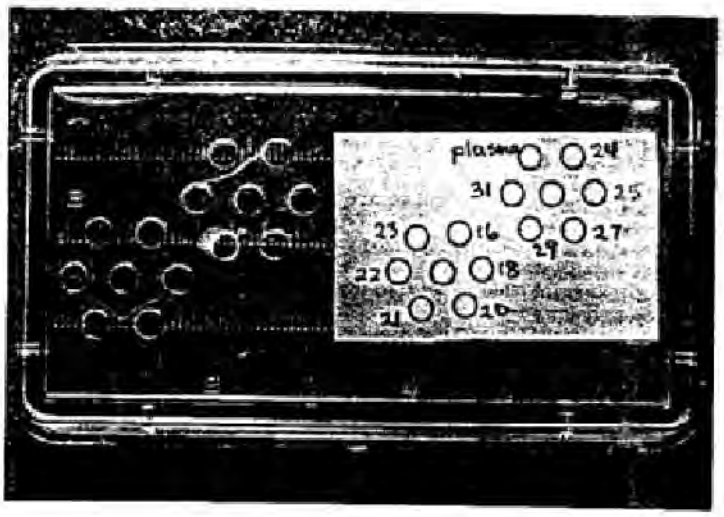


FIG. 2. Immunological identification of ceruloplasmin in column fractions by double immunodiffusion. Antibody made in rabbits against pure rat ceruloplasmin (20 μl) was placed in central well of each hexagonal rosette. Aliquots (20 μl) of fractions from Sephadex G-150 chromatography of rat plasma (Fig. 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

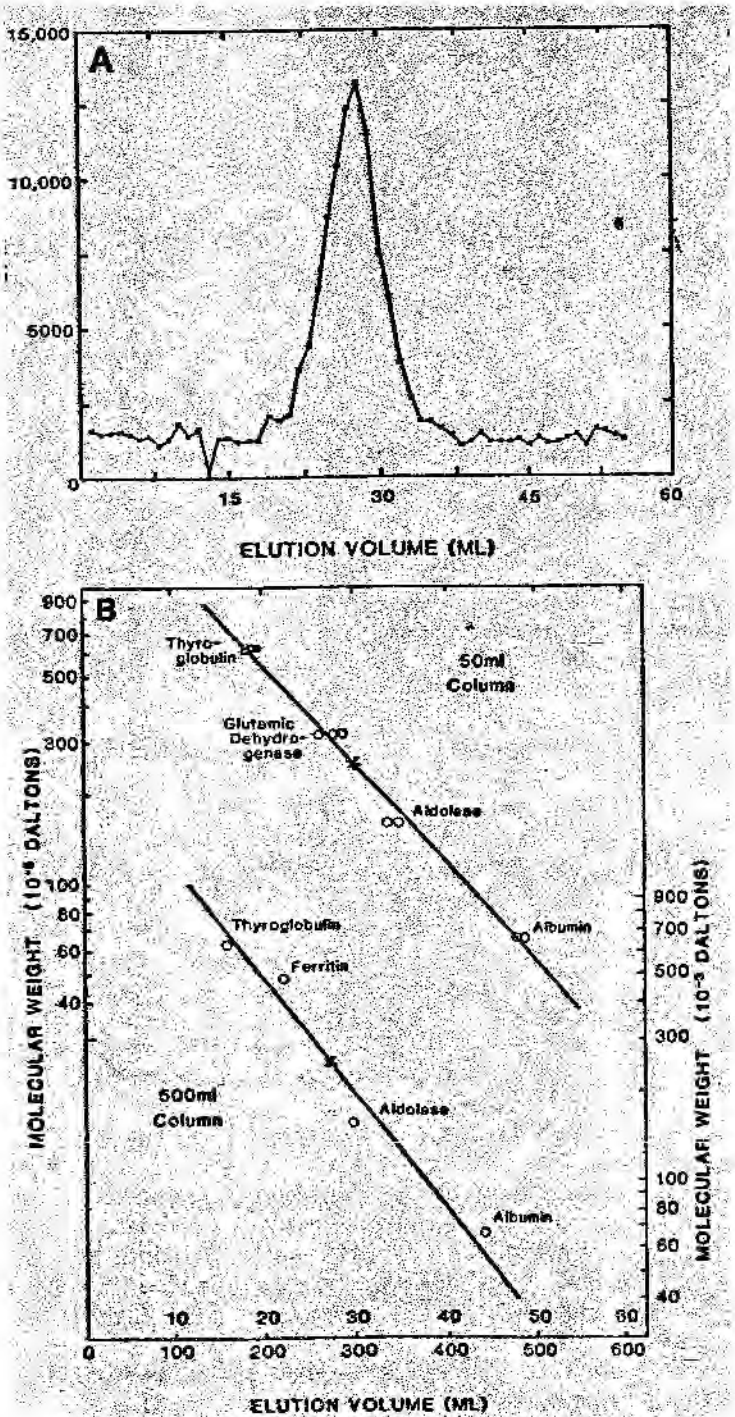


FIG. 3. Elution and apparent molecular weight of transcuprein, using Ultrogel AcA34. *A*: samples (2.5 ml) of ^{67}Cu-labeled transcuprein, obtained from chromatography of plasma on Sephadex G-150 (1st radioactive peak, Fig. 1A), were fractionated on 50-ml columns of Ultrogel AcA34. Elution of ^{67}Cu 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 (○). Stars (*) mark positions in which transcuprein samples eluted.

Figure A6.2(10)-4

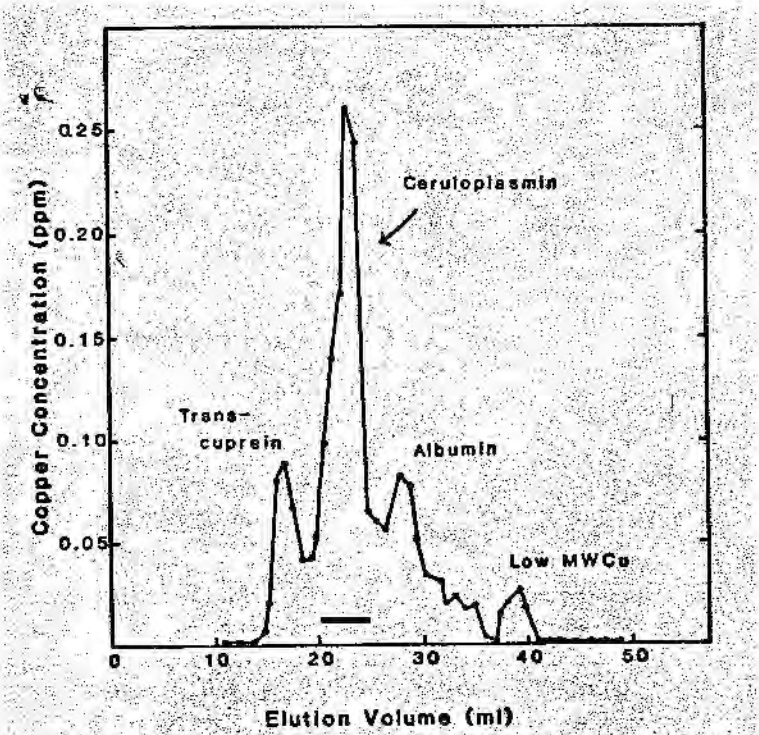


FIG. 4. Separation of copper components of plasma on Sephadex G-150. Samples (1.0 ml) of heparinized plasma from adult female rats were fractionated on 50-ml columns of Sephadex G-150 equilibrated with 20 mM phosphate. Fractions (1.0 ml) were assayed directly for copper content, by furnace atomic absorption 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

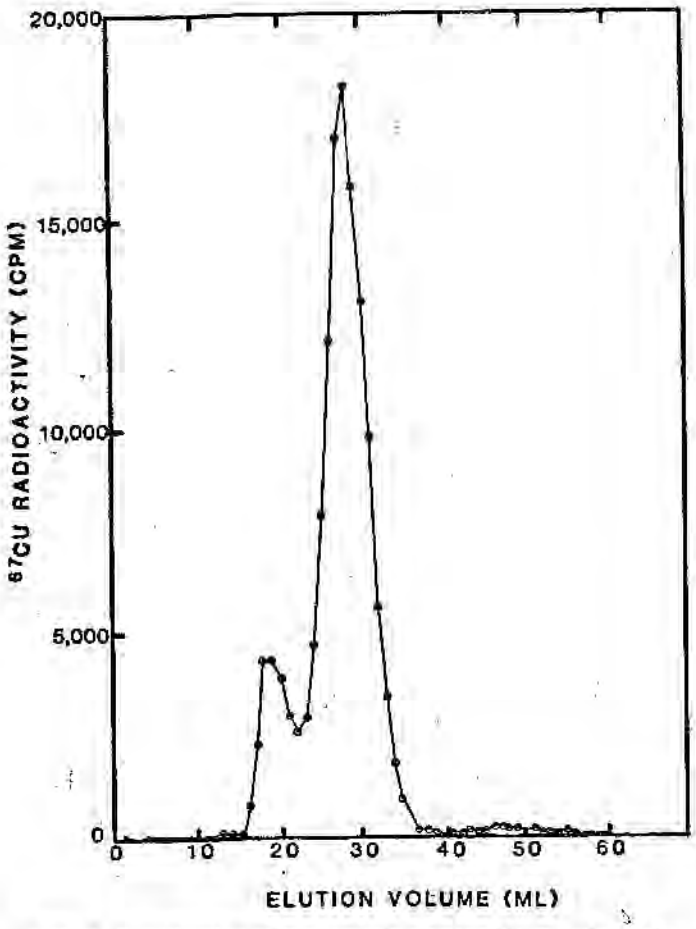


FIG. 5. Equilibration of transcuprein copper with albumin in vitro and vice versa. ^{67}Cu-labeled transcuprein (0.5 ml), obtained by fractionating plasma from rats injected with tracer $^{67}CuCl_2$ 15 min before death on columns of Sephadex G-150 (Fig. 1A), was mixed with an equal volume of cold plasma 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

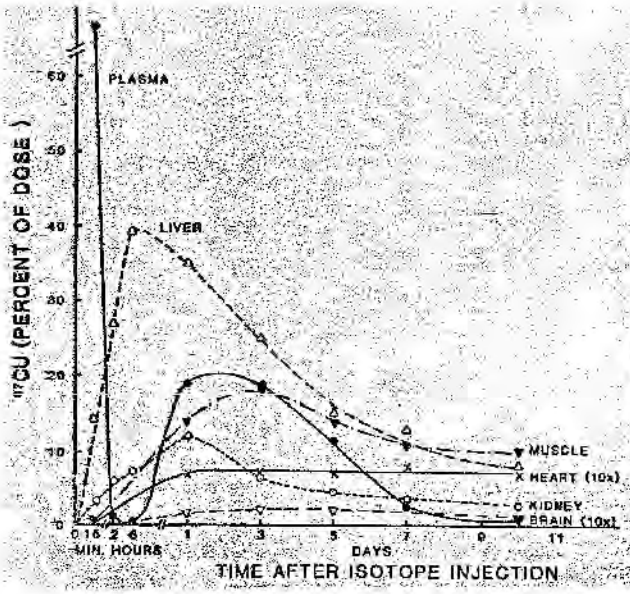


FIG. 6. Distribution of ^{67}Cu in rat tissues and plasma at various times after tracer administration. Radioactivity in plasma (●), liver (△), kidney (○), heart (×), muscle (▼), and brain (▽) was determined by counting whole organs or representative tissue samples from rats killed at various times after 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

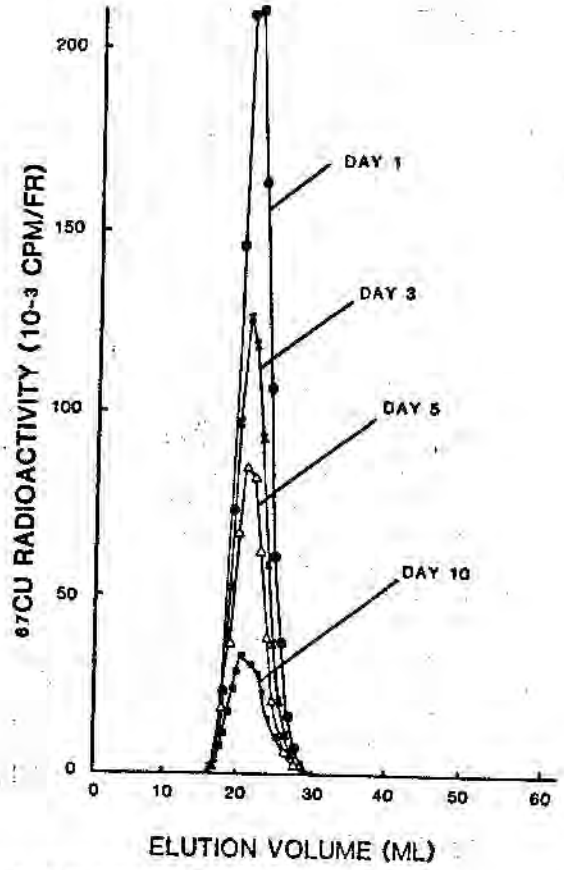


FIG. 7. Time course of ^{67}Cu-labeling of plasma components after 24 h. Samples of fresh plasma, taken from rats 1 to 10 days after administering $^{67}CuCl_2$ tracer, were fractionated on columns of Sephadex G-150 as described for Fig. 1. Rats killed at 1 (●), 3 (×), 5 (△), and 10 (□) days.

Figure A6.2(10)-8

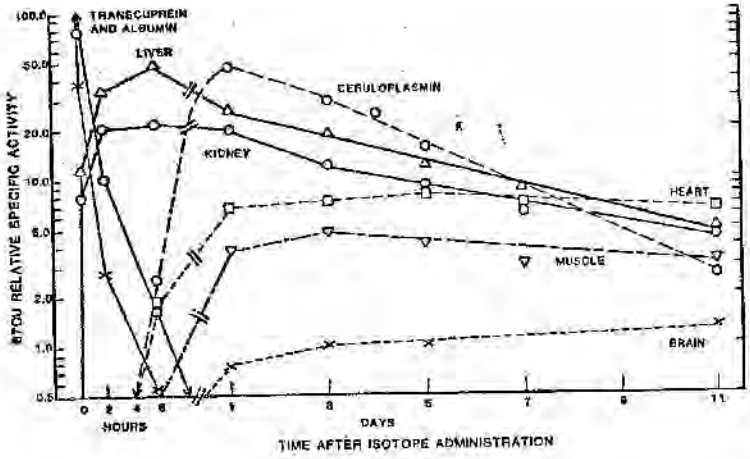


FIG. 8. Specific activity of co components and tissues at various ti after ^{67}Cu tracer administration. D for ^{67}Cu radioactivity (cpm/g or cp ml), relative to the value for liver at h, were recalculated as relative specifi activities of copper in these various com partments (cpm/μg Cu) based on tissue copper concentrations given in Table 3. Log specific activities of copper in albumin (o—o), transcuprein (×—×), liver (Δ—Δ), kidney (□—□), ceruloplasmin (○—○), 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
		59 REFERENCE	
59.1	**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	
		60 GUIDELINES AND QUALITY ASSURANCE	
60.1	**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")*	
60.2	**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)*	
60.3	**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")*	
		61 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.*	
61.1	**Test material**	$^{67}CuCl_2$	
61.1.1	Lot/Batch number	Not available	
61.1.2	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 5.0/11	**A6.2(11), Distribution of copper**

61.1.2.1 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.
61.1.2.2 Purity		*Give purity in % of active substance* [redacted]
61.1.2.3 Stability		*Describe stability of test material* See section 3.1.2.1.
61.1.2.4 Radiolabelling		*give structural location of radio labelling, give reason if not labelled* $^{67}Cu\ Cl_2$ (see section 3.1.2.1).
61.2	**Test Animals**	*Non*-entry *field*
61.2.1	Species	Rat
61.2.2	Strain	Sprague-Dawley
61.2.3	Source	Simonson Laboratories (Gilroy, CA) and Lab Pets (Riverside, CA).
61.2.4	Sex	Female
61.2.5	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.
61.3	**Procedures**	Non-entry field
61.3.1	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.
61.3.2 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.
61.3.3 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.
61.3.4 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.
61.3.5 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.
	62 RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are given below.*
62.1 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**.
62.1.1 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.
62.1.2 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.
62.1.3 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.
62.1.4 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.
62.2 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.

63 APPLICANT'S SUMMARY AND CONCLUSION

63.1 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}Cuceruloplasmin 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.
63.2	**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.
63.3	**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.
63.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
63.3.2 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	--	--	--	--	–	–

a Includes some rats sacrificed at 2 and 6 hr

Body and Organ Weights (g) of Experimental Anim

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

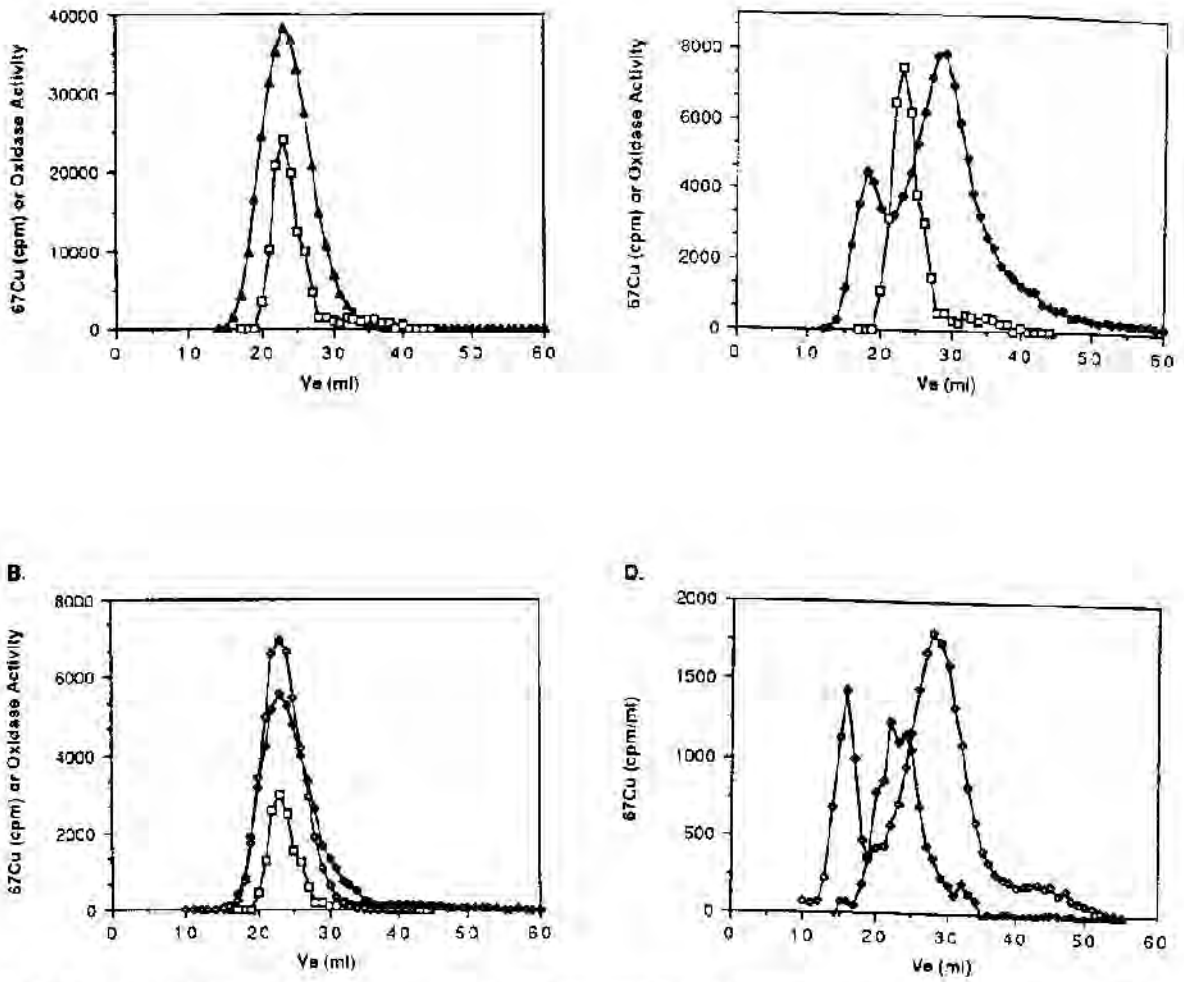


Figure 1. Gel chromatography of ^{67}Cu-labeled samples used for intravenous infusion, and serum samples from the maternal circulation taken 1 and 4 hr after infusion. One-milliliter samples were chromatographed on 50-ml columns of Sephadex G-150. Ceruloplasmin and albumin standards eluted at 23 ml and 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

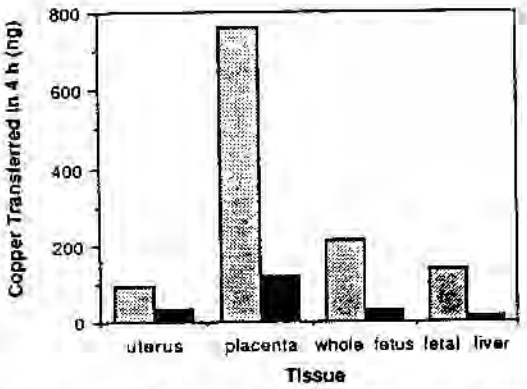


Figure 2. Estimates of actual nanograms of Cu transferred from the maternal circulation to pregnancy and fetal tissues over 4 hr, from ceruloplasmin (shaded bars) and from albumin + transcuprein (closed bars). Mean values for percentage of dose of radioactivity per organ (in Table II) and sizes of plasma 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

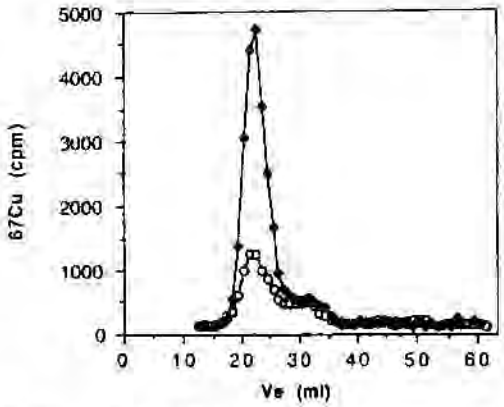


Figure 3. Effect of cycloheximide treatment on production of ^{67}Cu-ceruloplasmin by maternal tissues. Samples of plasma from pregnant rats injected intravenously with equal doses of ^{67}Cu(II) on albumin + transcuprein 4 hr before, were fractionated on Sephadex G-150, as in Figure 1. One rat received cycloheximide before and during the ^{67}Cu uptake period (O), 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

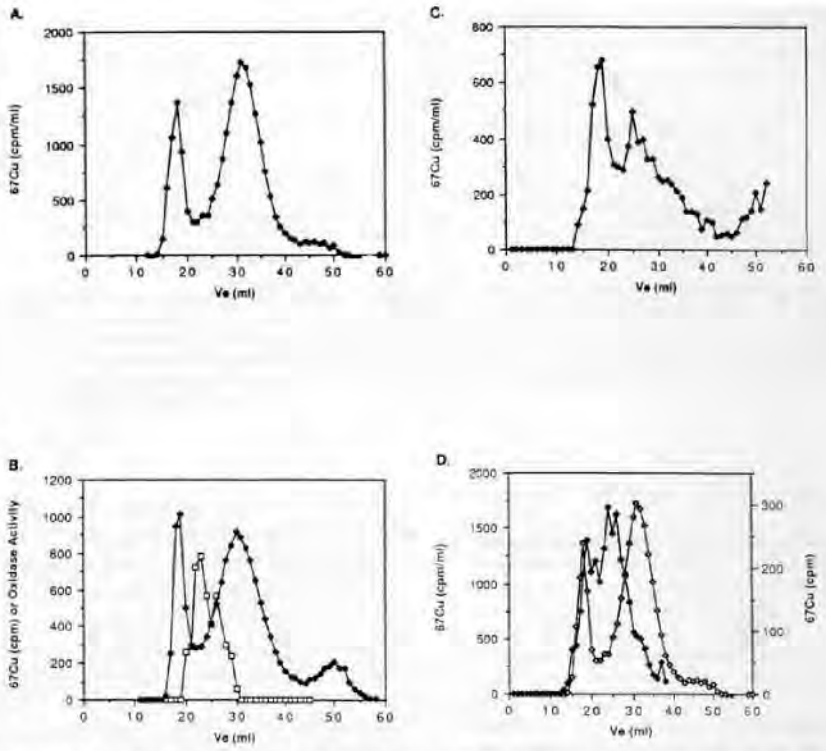


Figure 4. (A) Chromatography of fetal plasma samples taken at different times after infusion of ^{67}Cu components into the maternal circulation (as in Fig. 1). Fetal plasma from rats sacrificed 1 hr after infusion of ^{67}Cu-ceruloplasmin. (The same kind of chromatograph was obtained when ^{67}Cu-labeled 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

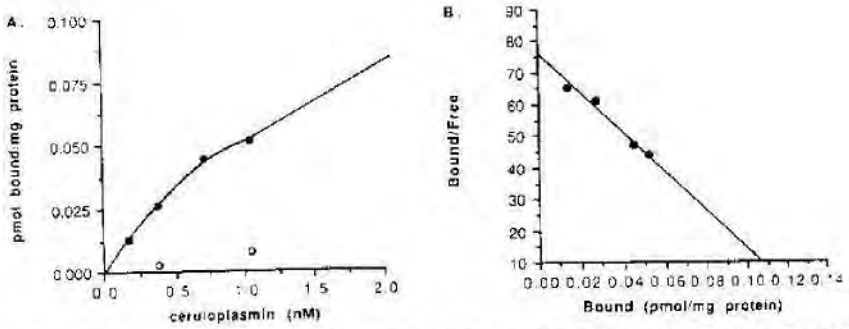


Figure 6. Binding of ^{67}Cu-ceruloplasmin to placental membranes. Aliquots of microsomal membranes from placentae of rats 1 day before term were incubated with various concentrations of ^{67}Cu-ceruloplasmin, partially purified from the plasma of a donor rat, for 2 hr at 25°C, before separation of free 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
		64 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	
		65 GUIDELINES AND QUALITY ASSURANCE	
65.1	**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")*	
65.2	**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)*	
65.3	**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")*	
		66 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.	
66.1	**Test material**	Samples of human sera were analysed for copper content.	
66.1.1	Lot/Batch number	Not applicable	
66.1.2	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**

66.1.2.1 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 NCIMayo 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.	
66.1.2.2 Purity	*Give purity in % of active substance* [redacted]	
66.1.2.3 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.	
66.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* Not necessary for a study of this type.	
66.2 Test Subjects	*Non*-entry *field*	
66.2.1 Species	Human	
66.2.2 Strain	Not applicable	
66.2.3 Source	See section 2.1.2.1	X
66.2.4 Sex	Male and female	
66.3 Procedures	Non-entry field	
66.3.1 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.	
66.3.2 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.

67 RESULTS AND DISCUSSION

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

67.1 Results

Non-entry field.

67.1.1 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).

67.1.2 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.

67.2 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.

68 APPLICANT'S SUMMARY AND CONCLUSION

68.1 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^{-3} 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.

68.2 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.
68.3 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.
68.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
68.3.2 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

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date [redacted]

Reference Materials and Methods [redacted]

Results and discussion [redacted]

Conclusion [redacted]

Reliability [redacted]

Acceptability [redacted]

Remarks [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)] [c]

[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.

[a] P<0.01 for difference from value for normal subjects.
[a] *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±6
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.

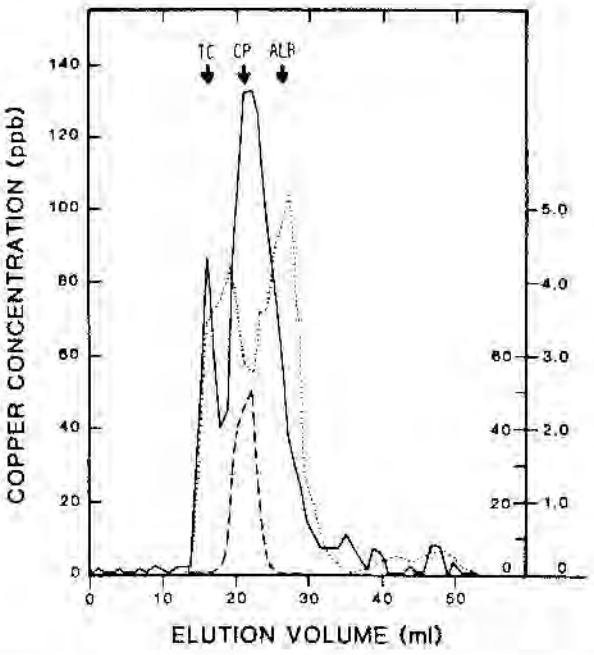
Table A6.2(12)-4

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	47 ± 2	50± 1
	(22)	(22)	(22)	(15)	(13)	(10)

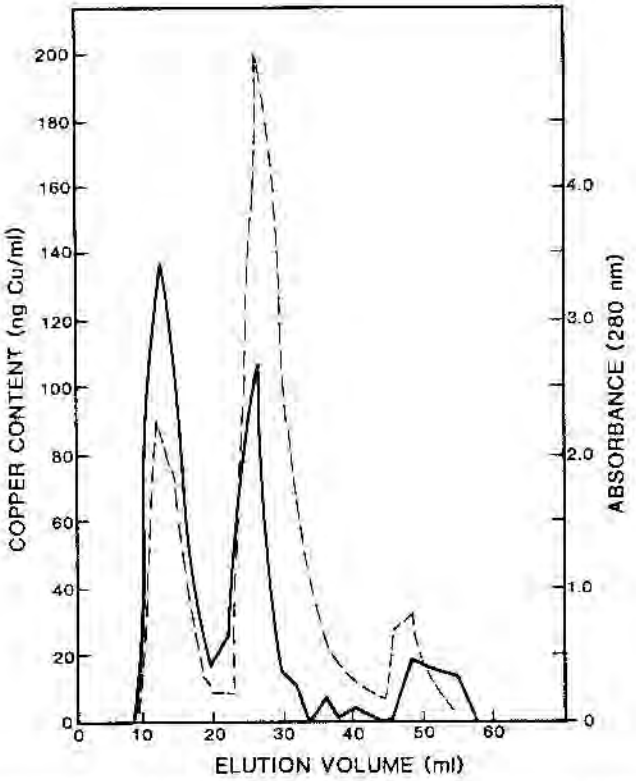
[a] *Numbers in parentheses* refer to small peaks; *dash* indicates no peak; *asterisk* indicates pronounced, very large peak.

Figure A6.2(12)-1



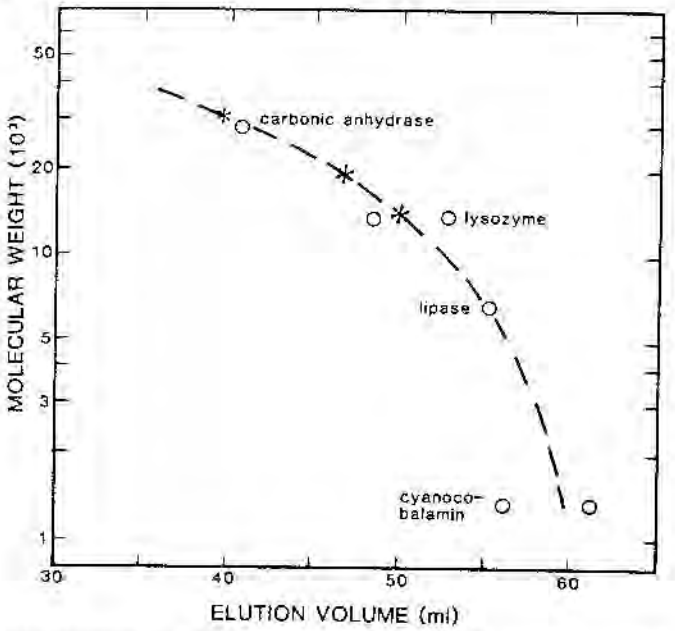
TEXT-FIGURE 1.—Fractionation of serum Cu components on columns of Sephadex G-150. Samples (1.0 ml) of fresh or previously frozen serum were applied to 50-ml gel columns, equilibrated with 0.9% NaCl. Fractions (0.9-1.1 ml) were collected and analyzed for Cu content by furnace atomic absorption spectroscopy (*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
		69 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	
		70 GUIDELINES AND QUALITY ASSURANCE	
70.1	**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")*	
70.2	**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)*	
70.3	**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
		71 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.*	
71.1	**Test material**	Cu^{2+} as $^{64}Cu(NO_3)_2$ and $^{67}Cu(NO_3)_2$.	
71.1.1	Lot/Batch number	Not available	
71.1.2	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**

71.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Not available
71.1.2.2 Purity	*Give purity in % of active substance* [redacted]
71.1.2.3 Stability	*Describe stability of test material* Not available
71.1.2.4 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).
71.2 Test Animals	*Non*-entry *field*
71.2.1 Species	Rat
71.2.2 Strain	Fischer
71.2.3 Source	Simonson Laboratories (Gilroy, CA).
71.2.4 Sex	Female
71.2.5 Age	3 – 4 months
71.2.6 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.
71.3 Procedures	Non-entry field
71.3.1 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.	
71.3.2 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.	X
71.3.3 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.	
71.3.4 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.	

72 RESULTS AND DISCUSSION

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

72.1 Results

Non-entry field.

72.1.1 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.

72.1.2 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.

72.1.3 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.

72.2 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 nonceruloplasmin 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 proteinlabelling, 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.

73 APPLICANT'S SUMMARY AND CONCLUSION

73.1 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
73.2	**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 µg/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 µg 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 µg/ml x plasma volume). This is in contrast with the 7.0 µg 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 µg 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 µg Cu from ceruloplasmin and 0.03-0.04 µg 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 µg 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.

73.3 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.

73.3.1 Reliability

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

2 Yes.

73.3.2 Deficiencies

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.)

Evaluation by Competent Authorities

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**

	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:	**Non-ceruloplasmin (tag Cu)**	**Ceruloplasmin (tag Cu)**	**(mg protein)**
Reference:			
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

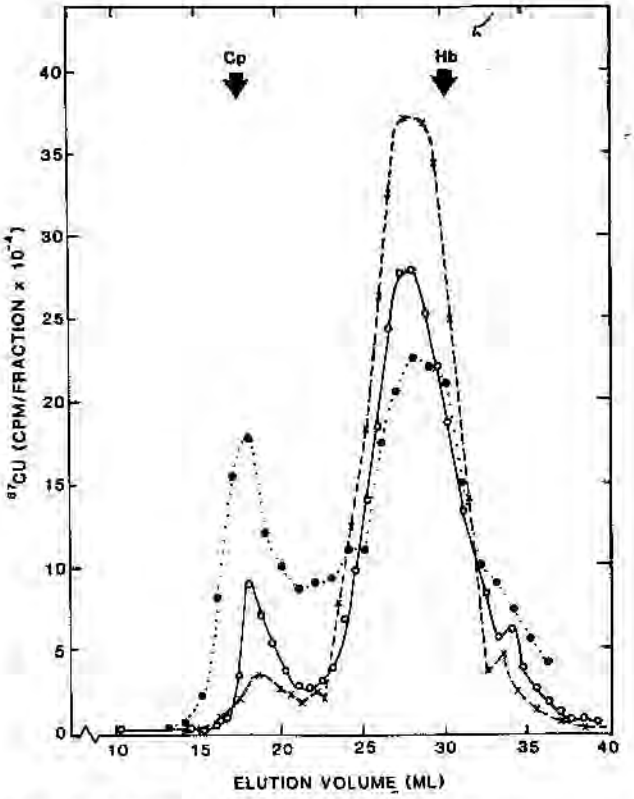


Fig. 2. Separation of ^{67}Cu-labeled ceruloplasmin and albumin by gel filtration. Samples of plasma (1.0 ml), from rats injected intraperitoneally with $^{67}Cu(NO_3)_2$ 2 h before death (●·····●), from rats intubated in the stomach with $^{67}Cu(NO_3)_2$ 10 min before death (o——o), or 'cold' plasma 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

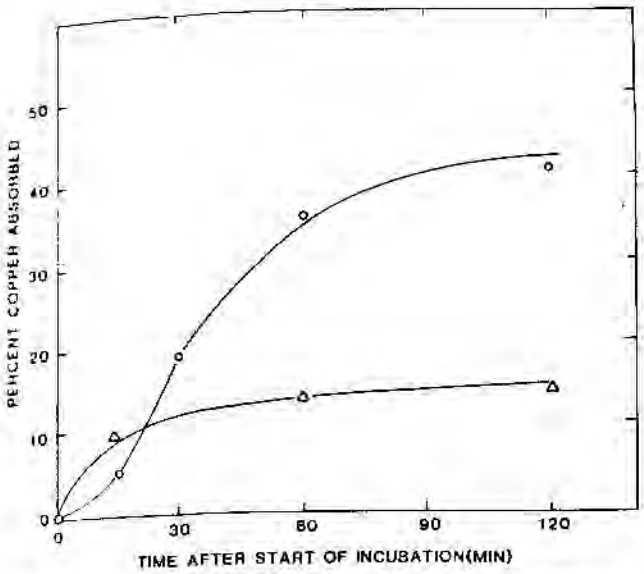


Fig. 3. Uptake of ^{67}Cu from ceruloplasmin and non-ceruloplasmin fractions of plasma by tumor cells in tissue culture. Sample (50 μl) of pooled material of the type obtained in Fig. 2 (●·····●) were added to 10^6 Ehrlich ascites tumor cells in 1.0 ml of nonserum-containing RPMI medium. Radioactivity 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
		74 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	
		75 GUIDELINES AND QUALITY ASSURANCE	
75.1	**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")*	
75.2	**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)*	
75.3	**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")*	
		76 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.	
76.1	**Test material**	Cu^{2+} as copper sulphate. ^{64}Cu in sodium acetate buffer.	X
76.1.1	Lot/Batch number	Not available	
76.1.2	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 5.0/14	**A6.2(14), Homeostasis of copper**

76.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* See section 3.1	
76.1.2.2 Purity	*Give purity in % of active substance* [redacted]	
76.1.2.3 Stability	*Describe stability of test material* Not available	
76.1.2.4 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	
76.2 Test Animals		
76.2.1 Species	Rats	
76.2.2 Strain	Wistar rats of the Hsd/Cpb strain.	
76.2.3 Source	Harlan-CPB, Zeist, the Netherlands.	
76.2.4 Sex	Male	
76.2.5 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.	
76.2.6 Number of animals per group	*Give number* *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.	
76.2.7 Controls	Yes	
76.3 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
76.3.1 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
76.3.2 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 **jt**mol 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 **jt**mol/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 **jt**mol/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 (λ) and the intercept, the % apparent retention (R_η), were computed. The biological half-life (T_b) was calculated as $T_b = \ln 2/\lambda$.	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.	
76.4 Examinations	Non-entry field	
76.4.1 Body weight	*yes/no (give time periods for determinations).* Yes. Bodyweight was monitored on days 0 and 42 of Experiment 2.	
76.4.2 Blood collections	*yes/no (give time periods for determinations).* Yes. Blood was collected for analysis of ceruloplasmin on day 42 of Experiment 2.	
76.4.3 Faeces collections	*yes/no (give time periods for determinations).* Faeces was collected quantitatively for analysis of Cu over the course of Experiment 1.	
76.4.4 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.	
	77 RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are*	

given below.

4.1 Results

Experiment 1: The data presented in **Table A6.2(14)-1** show that ascorbic acid administered by stomach tube simultaneously with ^{64}Cu significantly increased recovery of the dose in faeces. Within 1 day of administration, about 80% of the dose was found in faeces of ascorbic acid-treated animals and about 60% in that of non-treated animals.

Experiment 2: Growth and feed intake were similar in rats fed all of the diets tested (**Table A6.2(14)-2**). In rats fed the Cu-deficient diet, Cu status was impaired when compared with rats fed the Cu-adequate diets. In the former animals, Cu concentrations in all tissues were significantly reduced at 42 days. Plasma activity of ceruloplasmin was also decreased in rats fed the Cu-deficient diet (**Table A6.2(14)-2**).

Addition of ascorbic acid significantly reduced Cu concentrations in muscle and bone. The estimated whole-body content of Cu was decreased by 20% in rats that ingested ascorbic acid (**Table A6.2(14)-2**).

A day after i.p. administration of ^{64}Cu, distribution of the dose between tissues was different in rats fed the Cu-adequate and –deficient diets (**Table A6.2(14)-3**). The Cu-deficient diet caused Cu to accumulate preferentially in liver and muscle, and its excretion was depressed. In rats fed ascorbic acid, plasma and liver contained more and bone less radioactivity. Ascorbic acid inhibited excretion of radioactivity.

The time-course of body retention of the dose is shown in **Figure A6.2(14)-1**. In rats fed the Cu-deficient diet, the loss of the dose was diminished when compared with rats fed the Cu-adequate diet. Dietary ascorbic acid also reduced the rate of Cu-loss from the body. The biological half-life, as determined from body retention between days 2 and 4, was markedly prolonged in the Cu-deficient animals ($T_b = 31 \pm 3$ days) when compared with the control animals ($T_b = 12 \pm 4$ days) and the rats fed ascorbic acid ($T_b = 13 \pm 3$ days). Apparent Cu retention was significantly higher in both Cu-deficient animals ($85 \pm 7\%$) and those fed ascorbic acid ($88 \pm 4\%$), in comparison with control animals ($73 \pm 12\%$).

4.2	**Discussion**	This study showed that high amounts of dietary ascorbic acid impaired the Cu status of rats. In rats fed ascorbic acid, Cu concentrations of muscle and bone were significantly decreased. The estimated content of whole body Cu was lowered by 20%. However, a decrease in the dietary Cu concentration from 150 to 5 μmol/kg had a greater effect on Cu status than the addition of 1% (w/w) ascorbic acid to the diet. Ascorbic acid did not affect liver Cu. It appeared that ascorbic acid inhibited the intestinal absorption of Cu, increasing the recovery in faeces of orally administered ^{64}Cu (**Table A6.2(14)-1**). When gastric and intestinal absorption were circumvented by intraperitoneal administration of ^{64}Cu, tissue distribution and excretion of ^{64}Cu were altered similarly in Cu-deficient rats and animals fed ascorbic acid. However, in Cu-deficient animals, the biological half-life of ^{64}Cu was about twice that of controls, whereas in animals fed ascorbic acid, no increase in biological half-life was found. **Figure A6.2(14)-1** suggests that ascorbic acid induced an increased initial retention of Cu. Thus, although the kinetics are different, both Cu deficiency and ascorbic acid trigger mechanisms to reduce Cu loss from body stores to a minimum. This is illustrated by the inhibition of loss of radioactivity after i.p. administration of ^{64}Cu (**Table A6.2(14)-3**).
4.3	**Toxic effects, clinical signs**	*No effects / describe significant effects referring to data in results table* No effects.
5.4	**Recovery of labelled compound**	*state percentage* Not stated.

78 APPLICANT'S SUMMARY AND CONCLUSION

78.1	**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 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 was divided into 2 separate experiments. *Experiment 1*: Groups of 5 male Wistar rats of the Hsd/Cpb:WU strain were housed individually in a room with controlled temperature, relative humidity and lighting. Food and water were provided *ad libitum*. Recovery of orally administered radioactive Cu from faeces was determined using ^{64}Cu. Each rat received 0.8 jt mol/kg of ^{64}Cu in sodium acetate buffer by stomach tube, with or without 140 jtmol ascorbic acid. Faeces was collected to measure ^{64}Cu activity. *Experiment 2:* On day 0, rats were 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, rats were injected i.p. with a dose of ^{64}Cu. Whole body counting was performed by placing a container with the animal into a tank containing scintillation liquid (and equipped with a photomultiplier and a multichannel analyser). Whole body counting was performed 2 hours post-injection and the daily for 4 days. The measured whole-body

^{64}Cu activities at any time, R(t), and those measured 2 hours post-injection, R_0, were used to calculate 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 (λ) and intercept, and % apparent retention (R_η), were computed. Biological half-life (T_b) was calculated as $T_b = \ln 2/\lambda$.

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 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 typical values for the mass of these tissues. The specific activity was calculated from ^{64}Cu activity in Bq divided by Cu content in mmol.

^{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) by atomic absorption spectrometry. Ceruloplasmin in plasma was assayed as *p*-phenylenediamine oxidase activity. Cu analysis results were given as mean ± S.D. Statistical analyses were performed by the two-tailed Student's t-test; 0.05 was the maximum value for the type 1 error.

78.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Experiment 1: Ascorbic acid inhibited the intestinal absorption of Cu. Ascorbic acid administered by stomach tube simultaneously with ^{64}Cu significantly increased recovery of the dose in faeces. Within 1 day of administration, about 80% of the dose was found in faeces of ascorbic acid-treated animals and about 60% in that of non-treated animals.

Experiment 2: Growth and feed intake were similar in rats fed all of the diets tested. In rats fed the Cu-deficient diet, Cu status was impaired when compared with rats fed the Cu-adequate diets. In the former animals, Cu concentrations in all tissues were significantly reduced at 42 days. Plasma activity of ceruloplasmin was also decreased in rats fed the Cu-deficient diet.

Addition of ascorbic acid significantly reduced Cu concentrations in muscle and bone. The estimated whole-body content of Cu was decreased by 20% in rats that ingested ascorbic acid.

A day after i.p. administration of ^{64}Cu, distribution of the dose between tissues was different in rats fed the Cu-adequate and –deficient diets. The Cu-deficient diet caused Cu to accumulate preferentially in liver and muscle, and its excretion was depressed. In rats fed ascorbic acid, plasma and liver contained more and bone less radioactivity. Ascorbic acid inhibited excretion of radioactivity.

In rats fed the Cu-deficient diet, the loss of the dose was diminished when compared with rats fed the Cu-adequate diet. Dietary ascorbic acid also reduced the rate of Cu-loss from the body. The biological half-life, as determined from body retention between days 2 and 4, was markedly prolonged in the Cu-deficient animals (T_b = 31 ± 3 days) when compared with the control animals (T_b = 12 ± 4 days) and the rats fed ascorbic acid (T_b = 13 ± 3 days). Apparent Cu retention was significantly higher in both Cu-deficient animals (85 ± 7%) and those fed ascorbic acid (88 ± 4%), in comparison with control animals (73 ± 12%). It was concluded that both Cu-deficiency and ascorbic acid trigger mechanisms to reduce Cu loss from body stores to a minimum.

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**

78.3 Conclusion	Administration of ascorbic acid to rats fed a Cu-adequate diet caused a reduction in the whole-body content of Cu over a 6 week period. Also, 1 day after oral administration of ^{64}Cu, recovery of the dose in faeces was increased in rats fed ascorbic acid, suggesting that the vitamin depresses intestinal absorption of Cu. Furthermore, the rate of loss of ^{64}Cu administered by i.p. was decreased in rats fed ascorbic acid. These findings suggest that ascorbic acid induces a decreased efficiency of intestinal copper absorption, which in turn triggers mechanisms to preserve Cu in the body stores.
78.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
78.3.2 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 copper toxicokinetics. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

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]

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**
Acceptability	[redacted]
Remarks	

COMMENTS FROM ...

Date *Give date of comments submitted*

Table A6.2(14)-1. Effect of Ascorbic Acid on the Recovery of Orally-Administered 64Cu in Faeces (Experiment 1).

	Supplement	
	None, n = 5	Ascorbic acid, n = 5
	% of dose	
Day 1	62.9 ± 4.0	77.4[a] ± 4.0
Day 2	3.0 ± 3.4	4.8 ± 5.9
Day 3	1.5 ± 0.3	2.1 ± 1.2
Days 1 - 3	67.4 ± 5.7	84.3[a] ± 2.5

[a]P<0.05

Table A6.2(14)-2. Growth Performance, Tissue Copper Concentrations and Ceruloplasmin Activities (Experiment 2)*

Measure	Diet		
	Cu adequate, n = 4	Cu adequate + ascorbic acid, n = 8	Cu deficient, n = 4
Cu-intake, nmol/d	2.250	2.250	80
Ascorbic acid intake, µmol/d	--	850	--
Body weight, g, day 0	77 ± 2[a]	78 ± 3[a]	76 ± 3[a]
Body weight, g, day 42	290 ± 1[a]	289 ± 17[a]	287 ± 17[a]
Plasma Cu, µM	20 ± 3[a]	20 ± 3[a]	2 ± 1[b]
Liver Cu, nmol/g wet weight	69 ± 9[a]	66 ± 11[a]	18 ± 2[b]
Muscle Cu, nmol/g wet weight	22 ± 3[a]	17 ± 2[b]	10 ± 4[b]
Bone Cu, nmol/g wet weight	15 ± 4[a]	10 ± 3[b]	4 ± 1[b]
Estimated whole body Cu, µmol	4 ± 0.4[a]	3.3 ± 0.4[a]	1.5 ± 0.5[b]
Plasma ceruloplasmin, µM	3.6 ± 0.5[a]	3.8 ± 0.7[a]	0.7 ± 0.1[a]

* mean ± SD in each horizontal line common superscripts indicate P > 0.05.

Table A6.2(14)-3. ^{64}Cu Distribution (% Dose/tissue) and Specific Activities (SA) on Day 42, One Day after the Intraperitoneal Administration of ^{64}Cu (Experiment 2)*

Measure	Diet		
	Cu adequate, n = 4	Cu adequate + ascorbic acid, n = 8	Cu deficient, n = 4
Plasma, % Dose/tissue	11± 2[a]	17± 3[b]	8± 2[c]
Plasma, SA Bq/nmol	782± 103[a]	1164± 192[b]	8094± 1258[c]
Liver, % Dose/tissue	11± 1[a]	15± 2[b]	16± 1[b]
Liver, SA Bq/nmol	267± 46[a]	419± 44[b]	1655± 196[c]
Bone, % Dose/tissue	15± 1[a]	9± 1[b]	15± 1[a]
Bone, SA Bq/nmol	616± 137[a]	440± 122[b]	1684± 315[c]
Muscle, % Dose/tissue	10± 1[a]	10± 2[a]	13± 1[b]
Muscle, SA Bq/nmol	65± 8[a]	84± 9[b]	294± 45[c]
Carcass, % Dose	25± 3[a]	31± 5[b]	30± 3[b]
Carcass, SA Bq/nmol	297± 20[a]	418± 39[b]	897± 47[c]
Excretion, % Dose	28± 2[a]	18± 2[b]	18± 2[b]

* mean ± SD in each horizontal line common superscripts indicate P > 0.05.
[1]Calculated difference between the dose administered and the whole body activity 24 hours later.

Figure A6.2(14)-1

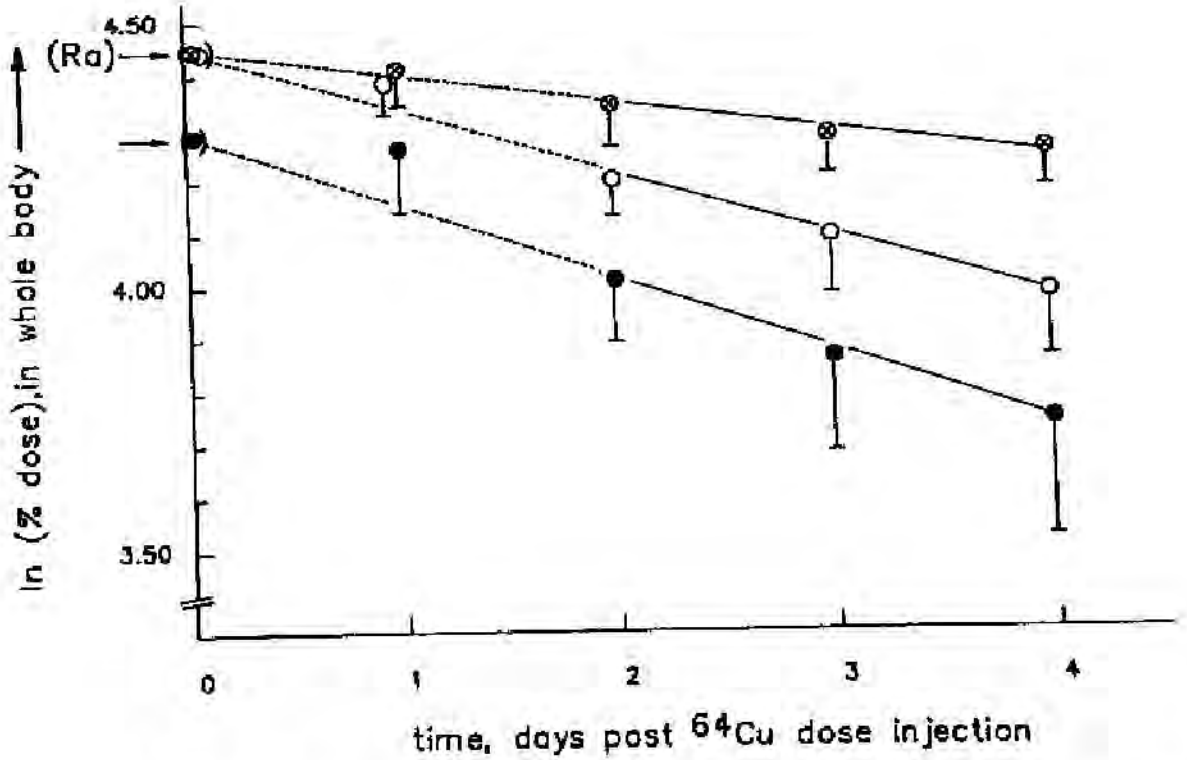


Fig. 1. Time course of whole body retention of injected ^{64}Cu. Symbols: ●, rats fed Cu-adequate diet (n = 4); ⊗, rats fed Cu-deficient diet (n = 4); ○, rats fed Cu-adequate diet supplemented with ascorbic acid (n = 8). Between brackets, the apparent retention (R_a) is shown.

			Official use only
		79 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).* Zhou, B. & Gitschier, J. (1997). h*CTR1*; A Human Gene for Copper Uptake Identified by Complementation in Yeast. Proc. Natl. Acad. Sci USA. **94**:7481-7486 (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	
		80 GUIDELINES AND QUALITY ASSURANCE	
80.1	**Guideline study**	No. This was a non-regulatory study carried out to investigate the molecular mechanism responsible for the high-affinity cellular uptake of copper in humans. No guidelines are available to address this objective. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
80.2	**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)*	
80.3	**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")*	
		81 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.*	
81.1	**Test material**	Refer to section 3.3	
81.1.1	Lot/Batch number	Not applicable	
81.1.2	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 5.0/15	**A6.2(15), Cellular and molecular metabolism of copper**
81.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Not applicable
81.1.2.2 Purity	*Give purity in % of active substance* [redacted]
81.1.2.3 Stability	*Describe stability of test material* Not applicable
81.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* Not appropriate to the current study.
81.2 Test Animals	*Non*-entry *field*
81.2.1 Species	Not applicable
81.2.2 Strain	Not applicable
81.2.3 Source	Not applicable
81.2.4 Sex	Not applicable
81.2.5 Age	Not applicable
81.3 Procedures	Non-entry field
81.3.1 Yeast transformation and DNA manipulation	The human cDNA yeast expression library was gifted by Massachusetts Institute of technology. For complementation studies, the library was transformed into a *ctr1* strain by using the lithium acetate method. Primary transformants recovered from SD-ura were then plated on yeast extract/peptone/glycerol or ethanol. Deletion and mutational analysis of *hCTR1* was achieved by PCR. Accuracy of all PCR-derived constructs was confirmed by sequencing.
81.3.2 Sequencing and sequence analysis	DNA sequencing was performed by both manual and automated sequencing by using the Sanger dideoxy method. BLAST, TBLASTX and TBLASTN were used to search the databases. The DNA STRIDER program was used for hydrophobicity analysis. Alignment was done using the University of Wisconsin Genetics Computer Group Package.
81.3.3 SOD1 assay	Yeast was grown in SD-ura liquid media, and cells were broken in lysis buffer by vortexing with glass beads. Protein concentration of the lysate was determined by the Bio-Rad DC protein assay with use of BSA as standard. SOD1 activity was assayed by a nitro blue tetrazolium test in polyacrylamide gel. Briefly, 10 μg of protein was loaded in each lane. The gel was soaked in colouring stain with gentle shaking in the dark for 10 min and developed on a light box. SOD1 activity was shown as a colourless band in the blue background. SOD1 activity was distinguished by its sensitivity to potassium cyanide at pH 9.
81.3.4 Atomic absorption spectrophotometry	The copper concentration of yeast lysates was determined by using a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer with pure copper as standard. hCTR1-UTR(F), hCTR1-UTR(R), and pDB20 transformants were all grown in SD-ura medium. Protein was obtained and concentration was determined as described for the SOD1 assay (section 3.3.3).
81.3.5 Copper and iron sensitivity assay	Yeast grown to OD 1.0 was used for a 10-fold serial dilution. A 5il volume was applied to each spot. Copper-rich plates were made by

	adding 1 M $CuSO_4$ to SD-ura media to a final concentration of 900 iM. Iron-limiting plates were made by adding 0.2 M batho-phenanthroline disulfonic acid disodium salt to SD-ura media to a final concentration of 50 iM.
81.3.6 Northern blot analysis and human cDNA Library screen	A CLONTECH multiple-human-tissue blot was probed with the h*CTR1* cDNA, stripped, and then reprobed with h*CTR2*. Both probes were made from the whole coding region. Randomly primed fibroblast and oligo(dT)-primed liver and placenta libraries were screened with h*CTR1* and h*CTR2* probes.
	82 RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are given below.*
82.1 Results	Non-entry field.
82.1.1 Isolation of a Human cDNA by complementation of the yeast copper uptake mutant *ctr1*	To clone the human *CTR1* homologue by functional complementation, a yeast *ctr1* strain was transformed with a human cDNA expression library and selected for growth on uracil deficient plates. About 2 million primary transformants were obtained, and these colonies were collected and pooled. As yeast *ctr1* cannot grow in non-fermenting media because of intracellular copper deficiency, a portion of the transformants pool was then plated on YPG or YPE plates for selection of a complementing human cDNA. After 7-10 days of growth, larger colonies were picked and analysed. Plasmids from 50 individual colonies were purified and retransformed into the *ctr1* strain. Five of these 50 clones were able to grow on YPG plates. The remaining 45 seemed to be false positives, because the original growth phenotype in the YPG(E) plate was not conferred by the harboured human cDNAs; these may have been yeast suppressor mutations. The transformed strain grows rather slowly on YPG compared with the wild-type *CTR1* strain, indicating that the recovered cDNA clones complement yeast *ctr1* inefficiently.
82.1.2 Sequence analysis of the cloned cDNA	Sequencing of the plasmids from the five positive clones indicated that they are from the same original clone because they have exactly the same length, the same sequence, and the same orientation. Translation in three reading frames of the potential transcript from the *adh* promoter (Padh) to the *adh* terminator (Tadh) revealed no reading frame longer than 100 aa. However, translation of all six reading frames indicated one potential product of 190 aa in the unexpected orientation, from Tadh to Padh. In addition to the significance of the derived amino acid sequence, several lines of evidence suggested that this reverse reading frame is authentic. First, there are several mammalian expressed sequence tags (ESTs) that have their 5'-to-3' direction corresponding to the isolated human cDNA from the Tadh-to-Padh direction. Second, a search against the STS database indicated that the cDNA portion adjacent to Tadh is associated with a human CpG island and, therefore, is presumably adjacent to the site of transcription initiation. Third, using the putative coding region to screen a cDNA library, several cDNAs with poly(A)$^+$ tails, all of which are located at the cDNA end adjacent to Padh. Finally reading from the Tadh-to-Padh direction , the first ATG of the ORF appears to be the authentic translational start site because the sequence is in good agreement with the Kozak consensus AXXATGG

and because there is an in-frame stop codon shortly upstream. It was therefore concluded that the authentic human transcript corresponds to the transcript from the Tadh-to-padh direction in the isolated clones.

Comparative analysis of the amino acid sequence derived from the ORF indicates that the human cDNA encodes a protein similar to *CTR1*. Both proteins are predicted to have three transmembrane domains by hydrophobicity analysis. Interestingly, h*CTR1* is significantly smaller than its yeast counterpart, mostly because the carboxyl terminus is truncated. At the amino terminus, h*CRT1* lacks significant primary sequence identity with *CTR1*, but they do share some common features. Both proteins are rich in methionine and serine at the amino termini, but hCTR1 is additionally abundant in histidines. The methionine-rich feature is observed in some bacterial copper-transport proteins, including the *Enterococcus hirae* P-type ATPase CopB and *Pseudomonas syringae* CopA and CopB. As both methionine and histidine are common ligands for copper, and as the amino-terminal domains of the CTR1 family of proteins are predicted to lie on the exracellular surface, these domains are likely to serve as scavengers for copper preceding copper uptake.

82.1.3 Analysis of the effects of the UTRs on expression in the yeast vector

Because the cDNA isolated by complementation was actually cloned in the reverse orientation and because growth of the complemented *ctr1* yeast on glycerol was far less robust than that of the wild-type yeast (**Figure A6.2(15)-1**), it was assumed that reorientation of the cDNA to the forward direction would result in a more efficient complementation. This hypothesis turned out to be incorrect, as complementation with the forward clone was actually no better than that with the reverse clone, as shown by growth at 12 days (**Figure A6.2(15)-1A**). As shown in **Figure A6.2(15)-1B,** significant sizes of colonies for wild-type *CTR-1* yeast were observed about 2 – 3 days later, whereas there was very limited growth for full-length h*CTR1* transformants in either orientation.

Further investigation of the effects on expression of the UTRs are summarised in **Table A6.2(15)-1**. Constructs that removed essentially all of the 3' UTR (about 1 kb) of h*CTR1* resulted in the same behaviour as that for the original cDNAs, i.e., bothe orientations complement and the reverse orientation works only slightly better. However, upon additional removal of the 5' UTR (about 150 bp), it was found that the construct with insert in the reverse direction no longer complements *ctr1*, but the construct with the insert in the forward orientation does. In fact , the forward 5'-UTR-less construct complements much more efficiently than the full-length original *hCTR1* in either orientation and restores growth comparable to that of the wild-type strain on a YPG plate. These experiments also confirm that complementation is due to the predicted hCRT1 protein that lies in the remaining 600 bp. To completely eliminate the possibility that the one short reading frame in the opposite orientation plays any role in *ctr1* complementation, a stop codon was introduced near the beginning of the ORF. This mutation also leads to a conservative substitution of serine for threonine at position 186 of h*CTR1*. The resulting construct still complements as well as hCTR1-UTR, demonstrating that the opposite reading frame does not play any role in the complementation. The result also indicates that the threonine-to-serine change does not affect hCTR1 activity. Based on these results, it is concluded that hCTR1 is the protein that affects the complementation of the yeast *ctr1* mutation. The UTR-less hCTR1 was therefore used in all following studies involving the

	expression of the h*CTR1*gene.	
82.1.4 Multiple defects of *ctr1* mutation complemented by h*CTR1* expression	The inability to use a nonfermentable carbon source is just one aspect of *ctr1* mutation. It is known that overexpression of CTR1 renders the transformed strain more sensitive to high levels of copper. A similar situation has been found for hCTR1: overexpression of *hCTR1* in either *CTR1* or *ctrl* background makes them more vulnerable to copper overload compared with vector-alone-derived strains (**Figure A6.2(15)-2A**). Another defect in *ctr1* strain is its inability to take up iron with high affinity because of defect in copper incorporation into FET3, an iron oxidase-reductase. This high-affinity iron uptake defect is also rescued by h*CTR1* expression, as shown in **Figure A6.2(15)-2B**. The control strain with vector alone cannot grow in SD-ura media in the presence of 50 µM bathophenanthrolinedisulfonic acid disodium salt, whereas the strains with *hCTR1* expression grow robustly. Expression of antisense (reverse orientation) UTR-less hCTR-1 has no effect as compared with the control (vector alone) in these assays. It is also well established that *ctr1* yeast is SOD1-deficient in the absence of surplus copper. To determine whether hCTR1 can rescue the SOD1 defect observed in *ctr1* yeast, SOD1 activity was tested in UTR-less hCTR1(F)-derived, vector-alone-derived, and UTR-less hCTR®-derived yeast *ctr1*strains. The UTR-less hCTR1(F)-derived strain has obvious SOD activity, whereas the other two have no detectable level of SOD activity (**Figure A6.2(15)-3A**). This antioxidant activity is inhibited by cyanide, indicating that it is from SOD1. The SOD1 defect in *ctr1* strain is thought to be as a result of low levels of cellular copper. By using atomic absorption spectrophotometry, it was shown that expression of hCRT1 does increase the cellular copper level in the *ctr1* background strain (**Figure A6.2(15)-3B**). This increase of the cellular copper level is thought to result in restoration of SOD1 activity in the *ctr1* mutant strain. There is no effect of *cCTR1* on growth on the *CTR1* strain compared with the controls (**Figure A6.2(15)-2A**).	
82.1.5 An homologous human gene, h*CTR2*	HCRT1 was used for a TBLASTN search against the EST database and a set of overlapping human ESTs that could code for a similar protein were found. Using this information, the full-length coding region of the related gene (now named h*CTR2*) was found by screening cDNA libraries. Although the majority of the h*CTR2* cDNAs had identical sequences, one alternatively spliced cDNA was aslo obtained. The rare, alternative transcript has one extra exon immediately after the h*CTR2* start codon, ATG, creating a premature translational stop; however, immediately after the stop codon, another ATG could be used to produce a product that id 56 ss longer, but is in-frame with h*CTR2*. Although the topology and length of hCTR2 are similar to that of hCTR1, the lack of obvious metal-binding motifs and the lower abundance of histidine ans methionine residues suggest that hCTR2 is more analogous to the yeast protein CTR2. This is thought to work as a low-affinity copper uptake protein based on two pieces of evidence. First, overexpression of *CTR2* makes yeast more sensitive to copper overload, and second, the *ctr2* mutant strain is more resistant to copper toxicity than the wild-type strain. To investigate the function of hCTR2 in terms of copper transportation, h*CRT2* was overexpressed in yeast	X

and the resulting strain was tested in a copper-replete plate. Compared with the strain transformed with vector alone, there was no obvious difference in terms of resistance to the toxicity of high copper levels. Expression of the whole minor form cDNA *hCTR2X* gave the same result.

82.1.6 Tissue distribution of expression of h*CTR1* and h*CTR2*

By Northern blot analysis of human tisssues, both *hCTR1* and *hCTR2* were found to be expressed in all organs abd tissues examined (Figure A6.2(12)-4). Liver exhibits the highest level of expression, whereas expression in brain and skeletal muscle is low.

HCTR1 has two major transcripts of approximately 2 and 5.5 kb and a less abundant transcript of about 8.5 kb. The ratios of these transcripts appear to be relatively constant in all organs/tissues, suggesting that they are under the same transcriptional control. The cDNA obtained in the complementation studies corresponds to the 2kb isoform. The *hCTR1* coding region was used to screen oligo(dT)-primed and randomly primed cDNA libraries in an effort to find any alternately spliced cDNA forms corresponding to the 5.5 kb mRNA, but none were recovered. The 3' UTR was not used to screen the cDNA library because it contains a CA repeat and an Alu repeat.

For *hCTR2*, a single major 2 kb transcript was observed, corresponding to the longest cDNA obtained. h*CTR2* is most abundantly expressed in the placenta. There was markedly reduced expression of h*CTR2* in the liver in contrast to h*CTR1*. Expression of n*CTR2* in the small intestine, colon mucosal lining, and peripheral blood leucocyte is also very low.

82.1.7 Localisation of h*CTR1* and *hCTR2* to 9q31/32

Examination of the STS and EST databases showed that both h*CTR1* and h*CTR2* have been mapped to 9q31/32. The 3' UTR of h*CTR1* contains a CA repeat marker (D9S262) that was placed in human 9q31/32. ESTs corresponding to h*CTR2* were place by the Massachusetts Institute of Technology Human Genome Project near markers also in 9q31/32. To determine whether *CTR1* and *CTR2* are adjacent genes, colocalisation in the same yeast artificial chromosome was tested. Yeast artificial chromosome clones 945D1 and 738F10 (Research Genetics) were reported to contain the D9S262 marker. It was confirmed that h*CTR1* is indeed located in these two yeast artificial chromosomes by DNA Southern blotting, but did not detect h*CTR2* in these two yeast artificial chromosomes, suggesting that h*CTR1* and h*CTR2* are not adjacent genes.

82.2 Discussion

Taking advantage of the inability of *ctr1* mutant yeast to grow on a nonfermentable carbon source, a human cDNA that codes for a putative copper transporter was isolated. It is proposed for the following reasons that h*CTR1* is a human high-affinity copper uptake gene. First, h*CTR1* can complement the yeast *ctr1* mutation, which is deficient for high-affinity copper uptake. h*CTR1* can rescue multiple aspects of defect in *ctr1* mutant: the inability to use nonfermentable carbon source; the iron transport defect; the SOD1 deficiency. Second, h*CTR1* expression increases yeast cellular copper concentration (as shown by atomic absorption) and makes yeast more vulnerable to copper overload. Third, hCTR1 and CTR1 are predicted to have similar transmembrane topology in addition to their sequence similarity, suggesting that complementation occurs at the same physiological level (copper transportation) instead of as, for instance, a downstream suppressor. Furthermore, hCTR1 has a copper binding domain that it shares with some bacterial copper transporters, suggesting that hCTR1 is directly

involved in copper homeostasis.

Although h*CTR1* can complement multiple defects of *ctr1* yeast, neither h*CTR2* nor its minor, alternatively spliced form h*CTR2X*, was functional in the yeast assay performed. However, it should be noted that the copper-resistance assay is not very robust. Furthermore, no efforts were made to optimise cDNA expression, as had been done with hCTR1. The possibility remains, therefore, that hCTR2 could be the mammalian counterpart of yeast CTR2.

It was noted that expression of h*CTR1* in yeast has a marginal effect in the copper-overload-sensitivity assay. One possible explanation is that the copper transport capacity of h*CTR1* in yeast is not high, although affinity may be high. A second possibility is that the rate limiting factor may not be h*CTR1* but some other factors in the pathway, for instance *FRE1*, which is negatively regulated by copper. Another explanation could be that h*CTR1* may not be perfectly compatible with other yeast components in the copper transport pathway.

The results of this study suggest that humans have at least one system for copper uptake homologous to that of yeast. It was found that hCTR1 was expressed in all tissues examined, suggesting that all tissues possess the ability to absorb free copper or copper conjugated to small molecules.

83 APPLICANT'S SUMMARY AND CONCLUSION

83.1 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 molecular mechanism responsible for the high-affinity cellular uptake of copper in humans. A human gene (h*CTR1*) involved in this process was isolated by complementation of the yeast high-affinity copper uptake mutant, *ctr1*. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP.

Yeast transformation, DNA manipulation and sequencing: For complementation studies, a human cDNA yeast expression library was transformed into a *ctr1* strain using the lithium acetate method. Primary transformants were then plated on yeast extract/peptone/glycerol or ethanol. Deletion and mutational analysis of h*CTR1* was achieved by PCR. Accuracy of all PCR-derived constructs was confirmed by sequencing. DNA sequencing was performed by both manual and automated sequencing.

The ability of the human gene to rescue iron transport and SOD1 defects in *ctr1* yeast was investigated.

SOD1 assay: Yeast cells were broken in lysis buffer by vortexing with glass beads and the protein concentration determined by the Bio-Rad DC protein assay using BSA as standard. SOD1 activity was assayed by a nitro blue tetrazolium test in polyacrylamide gel.

Copper and iron sensitivity assay: Yeast grown to OD 1.0 was used for a 10-fold serial dilution. A 5il volume was applied to each spot. Copper-rich plates were made by adding 1M $CuSO_4$ to SD-ura media to a final concentration of 900 iM. Iron-limiting plates were made by adding 0.2 M batho-phenanthroline disulfonic acid disodium salt to SD-ura media to a final concentration of 50 iM.

The ability of expression of the human gene in *ctr1* yeast to increase the

level of cellular copper was demonstrated by atomic absorption spectrophotometry (AAS).

Atomic absorption spectrophotometry: The copper concentration of yeast lysates was determined by using AAS with pure copper as standard. Transformants were grown in SD-ura medium. protein was obtained and its concentration determined as described for SOD1 assay.

The existence of an additional human gene (h*CTR2*) similar to h*CTR1* was investigated in a number of human tissue types.

Northern blot analysis and human cDNA Library screen: A multiple-human-tissue blot was probed with h*CTR1* cDNA, stripped, and then reprobed with h*CTR2*. Both probes were made from the whole coding region. Randomly primed fibroblast and oligo(dT)-primed liver and placenta libraries were screened with h*CTR1* and h*CTR2* probes.

83.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Isolation of a Human cDNA by complementation of the yeast copper uptake mutant ctr1: To clone the human *CTR1* homologue by functional complementation, a yeast *ctr1* strain was transformed with a human cDNA expression library and selected for growth on uracil deficient plates. As yeast *ctr1* cannot grow in non-fermenting media because of intracellular copper deficiency, a portion of the transformants pool that had been obtained in this way was plated on YPG or YPE for selection of a complementing human cDNA. Plasmids from 50 of the colonies grown were purified and retransformed into the *ctr1* strain. Five of these clones were able to grow on YPG plates. The remaining 45 were seemed to be false positives, as the original growth phenotype in the YPG(E) plate was not conferred by the harboured human cDNAs

Sequence analysis of the cloned cDNA: Sequencing of the plasmids from the five positive clones indicated that they were from the same original clone because they had exactly the same length (1.7 kb), sequence and orientation. Translation in three reading frames of the potential transcript from the *adh* promoter (Padh) to the *adh* terminator (Tadh) revealed no reading frame longer than 100 aa. However, translation of all six reading frames indicated one potential product of 190 aa in the unexpected orientation, from Tadh to Padh. It was considered that this corresponded to the authentic human transcript. Comparative analysis of the amino acid sequence indicated that the human cDNA encoded a protein similar to CTR1.

Analysis of the effects of untranslated regions (UTR) on expression in the yeast vector: Growth of the reverse-orientation complemented *ctr1* yeast on glycerol was less robust than that of wild-type yeast. Reorientation of the cDNA to the forward direction failed to result in more efficient complementation. However, investigation of the effects on expression of the UTRs showed that removal of the 3' UTR (about 1 kb) of h*CTR1* resulted in the same behaviour as that for the original cDNAs. Upon removal of the 5' UTR (about 150 bp), the construct with insert in the reverse direction no longer complemented *ctr1*, but the construct with the insert in the forward orientation did. In fact , the forward 5'-UTR-less construct complemented much more efficiently than the full-length original h*CTR1* in either orientation and restored growth comparable to that of the wild-type strain on a YPG plate. These experiments confirmed that complementation was due to the predicted hCRT1 protein that lies in the remaining 600 bp.

Multiple defects of ctr1 mutation complemented by hCTR1 expression:

Another defect in *ctr1* strain is its inability to take up iron with high affinity because of defective copper incorporation into FET3, an iron oxidase-reductase. This defect is rescued by h*CTR1* expression. The control strain with vector alone cannot grow in SD-ura media in the presence of 50 µM bathophenanthrolinedisulfonic acid disodium salt, whereas the strains with hCTR1 expression grow robustly.

ctr1 yeast is SOD1-deficient in the absence of surplus copper. To determine whether hCTR1can rescue this defect, SOD1 activity was tested in UTR-less hCTR1(F)-derived, vector-alone-derived, and UTR-less hCTR(R)-derived yeast *ctr1*strains. The UTR-less hCTR1(F)-derived strain has obvious SOD activity, whereas the other two have no detectable level of SOD activity.

The SOD1 defect in *ctr1* strain is thought to be as a result of low levels of cellular copper. Using AAS, it was shown that expression of hCTR1 increases the cellular copper level in the *ctr1* background strain. This increase of the cellular copper level is thought to result in restoration of SOD1 activity in the *ctr1* mutant strain.

An homologous human gene, hCTR2: The full-length coding region of a related gene (h*CTR2*) was found by screening cDNA libraries with hCTR1. Although the similar to hCTR1, the lack of obvious metal-binding motifs and the lower abundance of histidine and methionine residues suggested that hCTR2 was more analogous to the yeast low-affinity copper uptake protein CTR2.

Tissue distribution of expression of hCTR1 and hCTR2: By Northern blot analysis of human tisssues, both hCTR1 and hCTR2 were found to be expressed in all organs and tissues examined. Liver exhibited the highest level of expression, and brain and skeletal muscle the least.

h*CTR1* has two major transcripts of approximately 2 and 5.5 kb and a less abundant transcript of about 8.5 kb. The ratios of these transcripts appear to be relatively constant in all organs/tissues, suggesting that they are under the same transcriptional control. The cDNA obtained in the complementation studies corresponds to the 2kb isoform.

For h*CTR2*, a single major 2 kb transcript was observed, corresponding to the longest cDNA obtained. h*CTR2* is most abundantly expressed in the placenta. Expression of h*CTR2* in the liver, small intestine, colon mucosal lining, and peripheral blood leucocyte was low.

*Localisation of h*CTR1 *and* hCTR2 *to 9q31/32*: Examination of databases shows that both h*CTR1* and h*CTR2* have been mapped to 9q31/32. To determine whether *CTR1* and *CTR2* are adjacent genes, co-localisation in the same yeast artificial chromosome was tested. It was confirmed that h*CTR1* was located in two yeast artificial chromosomes by DNA Southern blotting, but h*CTR2* was not detected, suggesting that h*CTR1* and h*CTR2* are not adjacent genes.

83.3 Conclusion

A human gene (h*CTR1*) responsible for the celular uptake of copper was isolated by complementation of the yeast high-affinity copper uptake mutant, *ctr1*. As well as complementing *ctr1* growth defect on nonfermentable media, the human gene also rescues iron transport and SOD1 defects in *ctr1* yeast. In addition, expression of the h*CTR1* gene in yeast significantly increases the level of cellular copper. A second human gene, designated h*CTR2*, was identified in a database search. Both h*CTR1* and h*CTR2* were expressed in all human tissues examined.

83.3.1 Reliability

Based on the assessment of materials and methods include appropriate

	reliability indicator 0, 1, 2, 3, or 4 2
83.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of copper toxicokinetics. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

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]

COMMENTS FROM ...

Date	*Give date of comments submitted*