STABLE COPPER ISOTOPE INCORPORATION INTO SERUM CAERULOPLASMIN IN HUMAN HEALTH AND DISEASE

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ABSTRACT

Wilson's disease (WD) is a treatable inborn error of metabolism inherited in an autosomal recessive fashion (world-wide incidence 1:200,000). WD is a type of copper toxicity in which copper accumulation results from failure of normal liver transport into bile.

The identification of affected individuals within a known family is important to prevent irreversible neurological and/or hepatic damage. The determination of serum and urine copper and of serum caeruloplasmin concentrations are usually but not always sufficient to make a diagnosis.

A dynamic test to assay the incorporation of labelled Cu into serum caeruloplasmin may help diagnosis in equivocal cases. Tracer studies utilising the stable isotope ^{65}Cu offers numerous practical advantages over the expensive and short-lived ^{64}Cu (12hrs) and ^{67}Cu (72hrs) radio-isotopes. A method for the determination of ^{65}Cu in serum has been established using inductively coupled plasma mass spectrometry (ICP-MS). The pattern of ^{65}Cu incorporation into the plasma protein pool of normals, heterozygotes for the WD gene and cases at timed intervals after oral dosage has now been documented. Can these measurements be improved by isolation from plasma of the main plasma Cu containing protein, caeruloplasmin ? Fast protein liquid chromatography (FPLC; Pharmacia) was employed to separate the main ^{65}Cu containing plasma protein caeruloplasmin. Albumin was separated by gel filtration and affinity chromatography to varying degrees, subsequent advances in affinity media for Cp gave superior results.

Initially, four normal volunteers were given ^{65}Cu orally, and had blood samples withdrawn at intervals up to one month. The fresh serum was prepared for ICP-MS analysis of total ^{65}Cu and caeruloplasmin bound copper isolated. This thesis describes the development of methods to isolate the copper containing plasma protein caeruloplasmin labelled *in vivo* with ^{65}Cu.

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DECLARATION

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged

ABBREVIATIONS

Alb-Cu	-	Albumin-Copper complex
Cp	-	Caeruloplasmin
cpm	-	counts per minute
°C	-	degrees Celsius
ε-ACA	-	epsilon amino caproic acid
g	-	grams
g/L	-	grammes per litre
ICP-MS	-	Inductively Coupled Plasma-Mass Spectrometry
°K	-	degrees Kelvin
kDa	-	kilo Daltons
kHz	-	kilo Hertz
mA	-	mega Amps
mg	-	milligramme
mg/L	-	milligrammes per litre
mls	-	millilitres
mins	-	minutes
mmHg	-	millimetres Mercury
mm	-	millimetres
MHz	-	Mega Hertz
mV	-	millivolts
M	-	Molar
mM	-	millimolar
MW	-	Molecular Weight
nm	-	nanometres
nmol/L	-	nanomoles per litre
%En	-	percent enrichment ^{65}Cu over ^{63}Cu
PEG	-	Polyethylene glycol
PPD	-	para phenylene diamine
pH	-	acid to alkali scale 1-14
PBS	-	Phosphate Buffered Saline
pI	-	pH at which a protein has no net charge
rpm	-	revolutions per minute
s	-	seconds
µm	-	micrometre
µmol/L	-	micromoles per litre
µg	-	micro gramme
w/v	-	weight by volume

INTRODUCTION

This thesis is primarily concerned with the extracellular copper metabolism of man in healthy people and in a particular disease state known as Wilson's disease (Brewer 1992). Central to the research work were attempts to isolate quantitatively and qualitatively the plasma protein caeruloplasmin (ferroxidase, iron II : oxidoreductase)[EC 1.16.3.1], the novel aspect of the work being *in vivo* and *in vitro* experiments to label this protein with the stable isotope ^{65}Cu. Caeruloplasmin (MW 132kDa) is the major copper protein in serum accounting for up to 95% of serum copper with the remainder associated with albumin (MW 67kDa) and low molecular weight amino acids such as histidine. A defect in caeruloplasmin is not the root cause of Wilson's disease and people have been known to live indefinitely with no caeruloplasmin provided copper balance is achieved and maintained by other means. In fact, the gene for caeruloplasmin resides on chromosome 3 whereas the Wilson's disease gene is on chromosome 13. However, the question of copper balance in Wilson's disease cannot be addressed without consideration of caeruloplasmin's central role. Wilson's disease is characterised by failure to incorporate copper into caeruloplasmin in the liver, and failure to excrete copper from the liver into bile. The current understanding of the molecular basis of incorporation is that the Wilsons's disease gene produces the enzyme responsible for placing copper into caeruloplasmin. It is an ATPase of the cell membrane which binds copper in the reduced (Cu^{+}) state for which caeruloplasmin has an affinity. The route of copper excretion may have a similar dependence on the redox state of copper. The importance of this particular metal-binding ATPase is maintaining the copper ion in the reduced or cuprous state to allow incorporation into caeruloplasmin. Although much is known about the role of copper in many essential enzymes and it's transport by plasma proteins the mechanism of transport between tissues is unresolved.

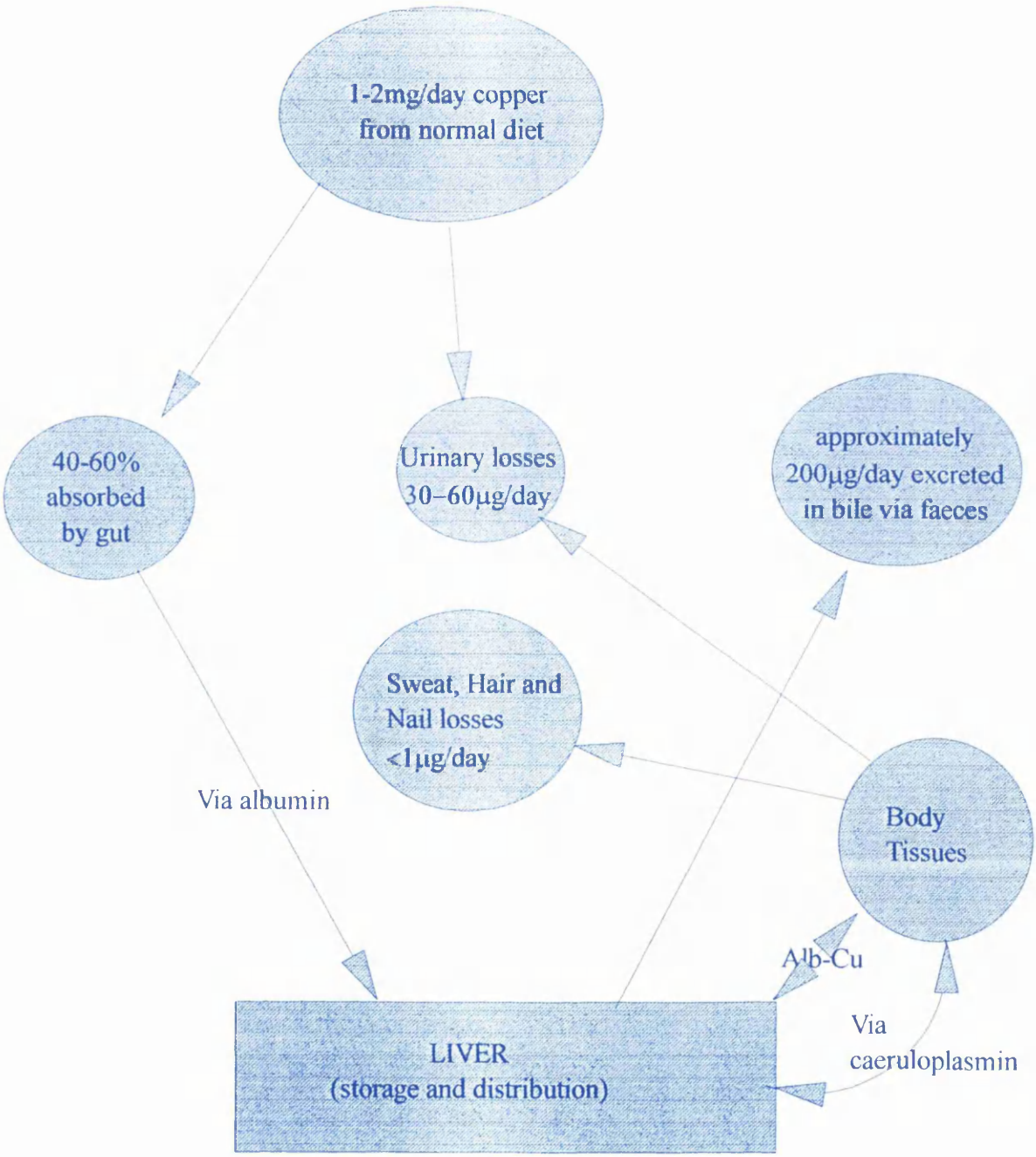
COPPER METABOLISM

The transition metal copper is an essential element required by all living systems. Classified as a "trace metal" copper is used by cells in the nanomolar range, but in micromolar concentrations may cause cell death (Shils 1991). Copper ions in living systems exist in two oxidation states: *cuprous* copper Cu^{+} and *cupric* copper Cu^{++}. The *cupric* ion is an oxidising agent and blue in colour whereas the *cuprous* ion is colourless.

In man, copper ingested in the diet enters the gut and is first associated with albumin. Albumin has a single high affinity binding site for copper but readily donates this copper to the liver. (Linder et al. 1991, believe this function to be carried out by a high molecular weight protein designated "transcuprein" although this is not fully accepted). In the hepatocytes copper is associated with metallothionein. The liver functions as a store for copper from where it can be transported on caeruloplasmin to the tissues or excreted surplus to requirement as undegradable complexes in bile (Diag. 1). This prevents reabsorption in the gut and allows excess copper to leave the body via faeces at the approximate rate of 200μg daily. Urine represents a minor route of excretion with daily output of 30-60μg copper. Small amounts are also lost in sweat, hair and nails.

The primary role of copper is as a component of the many intracellular copper metalloenzymes which perform various metabolic functions. Many cuproenzymes share a common biochemical principle by which molecular oxygen or a related species such as superoxide radicals are consumed. Cytochrome *c* oxidase governs the terminal reaction in mitochondrial oxidative phosphorylation. Lysyl oxidase is the key enzyme for the cross-linking reactions for collagen and elastin in connective tissues. Dopamine-β-hydroxylase metabolises catecholamines in the brain, superoxide dismutase is thought to provide antioxidant protection.

NORMAL COPPER BALANCE IN MAN

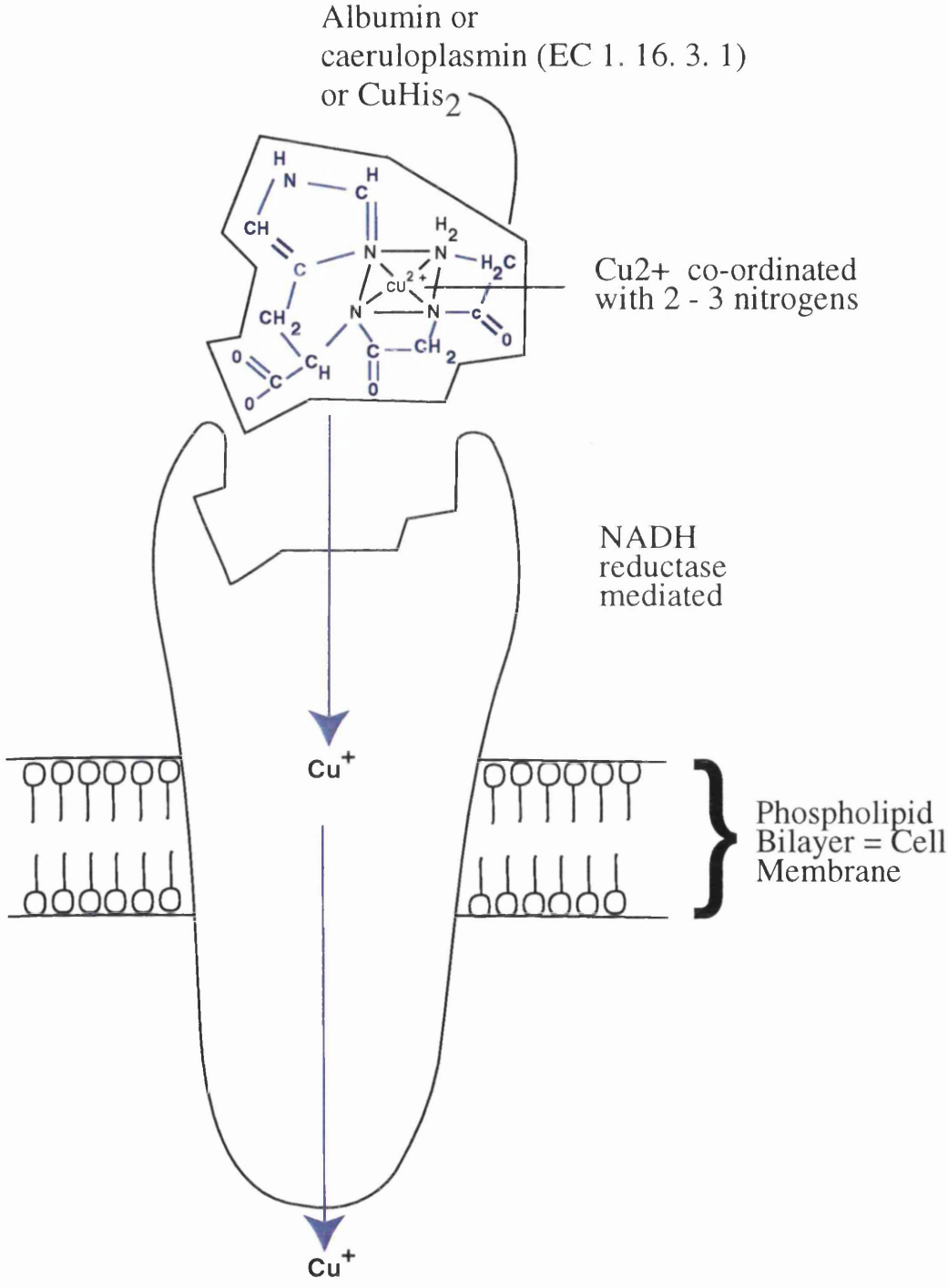


Copper incorporated into caeruloplasmin and released into the bloodstream is free to donate its copper where the requirements of a cell provide the conditions of release (Diag. 2 after McArdle 1992). Caeruloplasmin is also responsible for catalysing the oxidation of *ferrous* (Fe^{++}) to *ferric* (Fe^{+++}) ions.

WILSON'S DISEASE

Wilson's disease is a rare (1:200,000) inborn error of metabolism that results in abnormal copper deposition in several organs. Excessive copper retention and toxic accumulation in the liver, kidney and basal ganglia of the brain leads to cirrhosis, renal tubular disorders and neurological damage. Characteristic biochemical findings are low serum copper <5μmol/l (ref. interval: 12-24μmol/l), increased urinary copper >2μmol/24hrs (ref. interval <1μmol/24hrs) and low caeruloplasmin concentrations (ref. interval: 200-500mg/l). Given positive indications from these tests a liver biopsy would be performed to determine the degree of copper accumulation, values of liver copper >250μgCu/g dry wt are significant. Wilson's disease is treatable to give normal lifespans when diagnosis is made at an early stage (Hiyamuta 1993). Treatment is by a variety of chelating agents that bind copper and allow excretion in urine, or by using zinc to block copper absorption in the gut. The gene for Wilsons's disease is located on chromosome 13 and closely linked markers can be used to follow the inheritance of the disease in affected kindreds. Restriction Fragment Length Polymorphism (RFLP) mapping uses markers on either side of the gene (Gaffney 1992, Houwen 1993). This technique is slow and labour intensive also the distance of the markers from the gene makes "crossovers" possible. Presymptomatic diagnosis can be made more rapidly with Polymerase Chain Reaction (PCR) techniques using closer CA-repeat markers which are within a 300 kilo-base region of the gene (Thomas 1994).

A MODEL FOR COPPER UPTAKE BY MAMMALIAN CELL



The gene product proposed is a P-type ATPase, with six metal binding regions and this protein is expressed in liver and kidney (Bull 1993, Petrukhin 1993, Tanzi 1993, Petrukhin 1994).

AIMS

(1) To determine the pattern of ^{65}Cu incorporation into the plasma protein pool at timed intervals after oral dosage.

(2) To develop chromatographic separations of the main ^{65}Cu containing plasma protein fractions at different times after oral dosage.

(3) To compare the time course of Cu isotope appearance in the serum of healthy controls with those of Wilson's disease kindreds.

(4) To examine the intestinal uptake and excretion of ^{65}Cu in healthy controls and in patients with intestinal malabsorption.

CAERULOPLASMIN

Caeruloplasmin (Cp) is a single-chain α-2-glycoprotein of 1,046 amino acids which binds up to 95% of serum copper (Saenko 1994). The molecular weight is 132 kDa and this cleaves readily into three fragments of 67, 50 and 19 kDa by autolysis (Bonaccorsi de Patti 1992). The pronounced lability of Cp has led to the hypothesis that these fragments could possess specific functions (Harris 1993). The protein contains 6-7 copper atoms in three distinct classes of copper binding site which are required for overall functional integrity. Type 1 is a mononuclear intensely blue site with an optical absorption at 610 nm. Type 2 or 'non-blue' copper has no visible absorption and Type 3 copper is likely to be made from two spin-coupled Cu^{++} ions absorbing weakly at 330nm (Calabrese 1989, Rylkov 1991).

BIOLOGICAL PROPERTIES

Caeruloplasmin possesses several primary functions as a copper transporter, as an enzyme (Holmberg 1951) and as a plasma antioxidant (Goldstein 1979, Sergeev 1993). Caeruloplasmin exhibits oxidase activity towards certain aromatic amines and phenols, cystine and ascorbic acid although the physiological significance of this is not known. Caeruloplasmin controls *ferrous* iron oxidation, catalysing the incorporation of absorbed Fe^{++} into the iron transport protein transferrin (Gutteridge 1993). Serum Cp levels rise in response to tissue injury, infection and inflammation as part of the acute-phase response, Cp levels doubling or tripling in the days following the event (Hansen 1988). The elevated total serum copper found in certain cancers was found to be due to Cp copper and independent of ultrafiltrable copper (Chan 1993).

PREVIOUS ISOLATIONS

Caeruloplasmin was first isolated in 1948 by Holmberg and Laurell using salt and ethanol precipitation (Cohn fractionation). Subsequent developments in chromatographic methods established anion exchange as the principle technique for the separation and purification of Cp. A crude DEAE cellulose column with sodium actate buffer was one of the first methods to quantify Cp in health and disease (Deutsch 1960). A large-scale method succeeded in separating two forms of Cp from apocaeruloplasmin and degraded Cp. One hundred litres of plasma was fractionated with polyethylene glycol (PEG 4000) and then further separated on DEAE-Sephacel (cellulose based), hydroxyapatite and Sephadex G-200 (dextran based) to produce a homogeneous protein with maximal enzymatic activity (Noyer 1980). A more modern method used DEAE-Sephadex and Sephadex G-200 to purify chicken Cp which is present at much lower concentrations than in man (Disilvestro 1985). The first use of FPLC employed DEAE-Sepharose and Superose 12 (both agarose based) for the purification of rat Cp (Ryan 1992). The counter ion used for anion exchange is Cl^- from sodium chloride. The buffers used vary. Mostly used are Tris-HCl and phosphate buffers including phosphate buffered saline (PBS). Acetate buffer is used for storage of the separation products. Phosphate buffers are within the range pH 6.5-7.5 and acetate buffers pH 5-6. The stability of isolated Cp is improved by cold storage in isotonic solution and adding protease inhibitors. The protease inhibitor epsilon-amino caproic acid (ε-ACA) is used at a 20mM concentration.

FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC)

The FPLC apparatus consists of two calibrated pumps (P-500) controlled by a programmable gradient controller (G-250). A mixing chamber followed by a pre-filter is linked to a three-way valve. Attached to the three-way valve is a 10ml sample applicator (Superloop), the chromatography column and the waste bottle. The column outlet leads to an ultra violet monitor (UV-1), and from there to a carousel fraction collector (FRAC-1). A chart recorder traces the absorbance of eluent passing through a quartz cell at 280nm.

SAMPLE HANDLING

Freshly withdrawn blood samples in plain polypropylene tubes were allowed to clot for 15 minutes. After centrifugation at 3,000 rpm for 15 minutes the clear yellow serum was removed with 1ml disposable pipettes. The protease inhibitor ε-ACA was added as a 1M solution to give a final concentration of 20mM before storage at 4°C. Serum, prior to chromatography was diluted 1:1 with buffer (1 X PBS + 20mM ε-ACA) and passed through a 0.45μm filter (Millipore) fitted to a disposable syringe (Becton Dickinson). This was done to reduce the viscosity and ionic strength of the sample and to remove any fibrin which may have formed.

The PBS buffer used was purchased as a single batch of concentrate (Gibco; Paisley). After dilution with high purity water (Elgastat) and addition of ε-ACA, the pH was 6.8 to 6.9. The buffer was prepared without calcium and magnesium to avoid ionic interactions.

Bacterial contamination was reduced by use of sterile plastic, such as polypropylene and single-use, non-bioadhesive filtration membranes (Nanosep, Amicon, Sherwood, Sterilin).

MEASUREMENTS

The determination of copper in serum and column fractions was performed by graphite furnace and flame atomic absorption spectrometry (Perkin Elmer, GBC) or by inductively coupled plasma atomic emission spectroscopy (Thermo Jarrel Ash ATOMSCAN25), (methods 5 and 6 respectively). Spectrophotometers (Philips, Packard) were used to measure absorbance at 280 and 610nm and the ratio 610/280nm used to gauge the purity of the isolated fractions. A ratio of 0.045 or better, indicates that a sample contains 90% pure Cp. This was confirmed by high resolution electrophoresis (Sebia), (method 4). Spectrophotometry was also used to determine the specific oxidase activity of Cp using as substrate, p-phenyldiamine (PPD), and measuring the rate of formation of the purple oxidised product (method 12). Immunological reactivity was assessed using immunodiffusion with polyclonal antibodies (Behring), (method 7).

GEL FILTRATION

INTRODUCTION

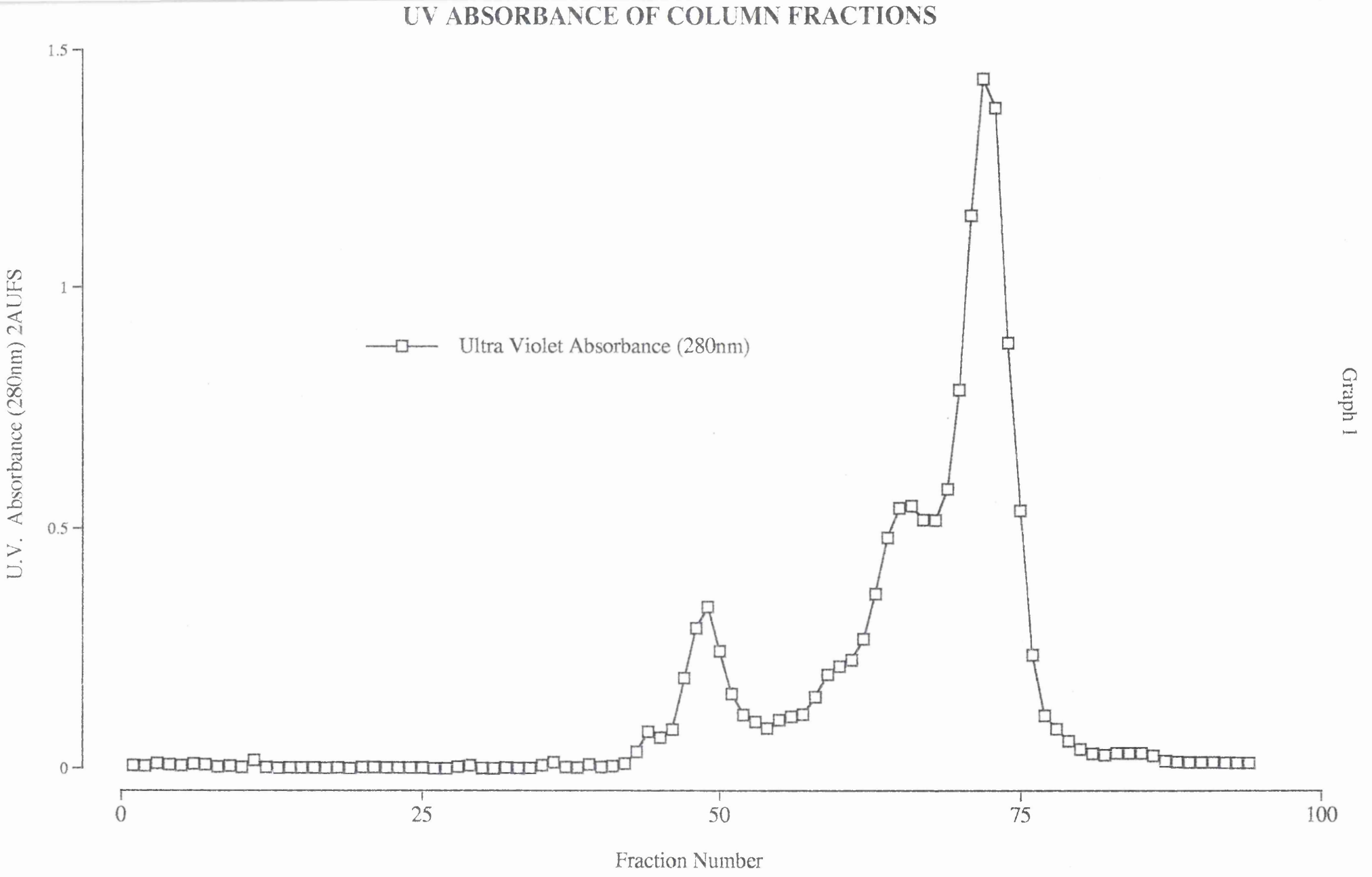
Gel filtration is a technique for the separation of proteins according to size. The gel is comprised of porous beads, packed into a chromatography column, through which a liquid phase is pumped. The separation depends on the different abilities of the proteins to enter and leave the pores within the beads. The largest proteins cannot enter even the biggest pores and hence pass through the column fastest and are eluted first. Smaller proteins enter the pores to different degrees depending on their size and shape, while the smallest proteins are retarded by the labyrinth of channels in the gel and elute last. The best resolution is achieved using a small sample applied to a long column at a slow flow rate.

MEDIA SELECTION

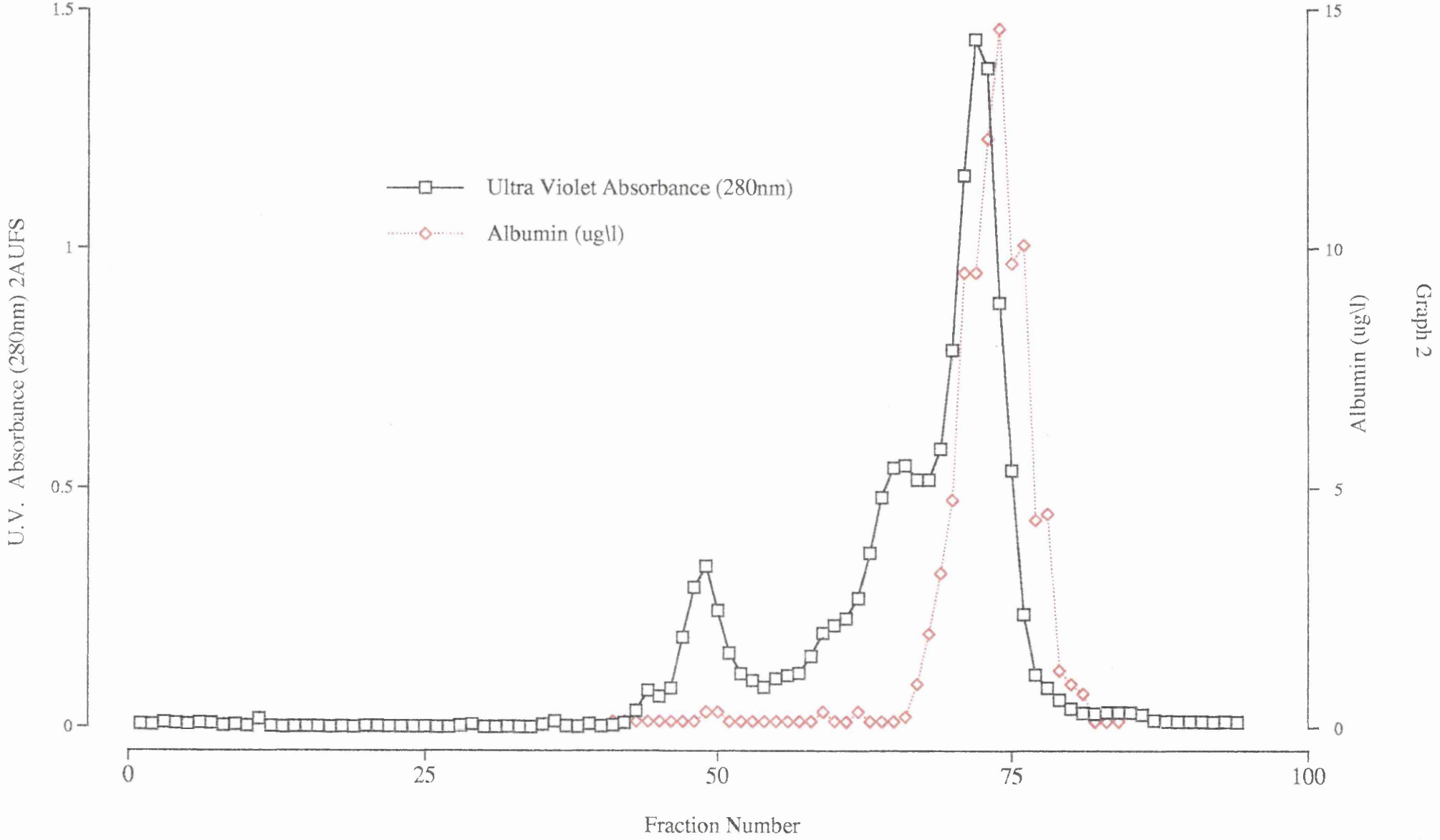
The copper containing plasma proteins are best separated with a gel having a range of pore sizes which encompass the entire fractionation range of the (more than a hundred) serum proteins. Sephacryl 300HR (Pharmacia) fits this criteria with a fractionation range of 10 -1,500kDa. Sephacryl also has the high mechanical strength necessary to support a long column without causing compression of the gel bed. The composite gel is made by covalently cross-linking allyl dextran with N,N'-methylene bisacrylamide. It is the dextran component which contributes to the porosity of the gel and hence its fractionation range. The gel is supplied pre-swollen with a wet bead diameter of 25-75μm in a slurry of 20% ethanol.

RUN CONDITIONS

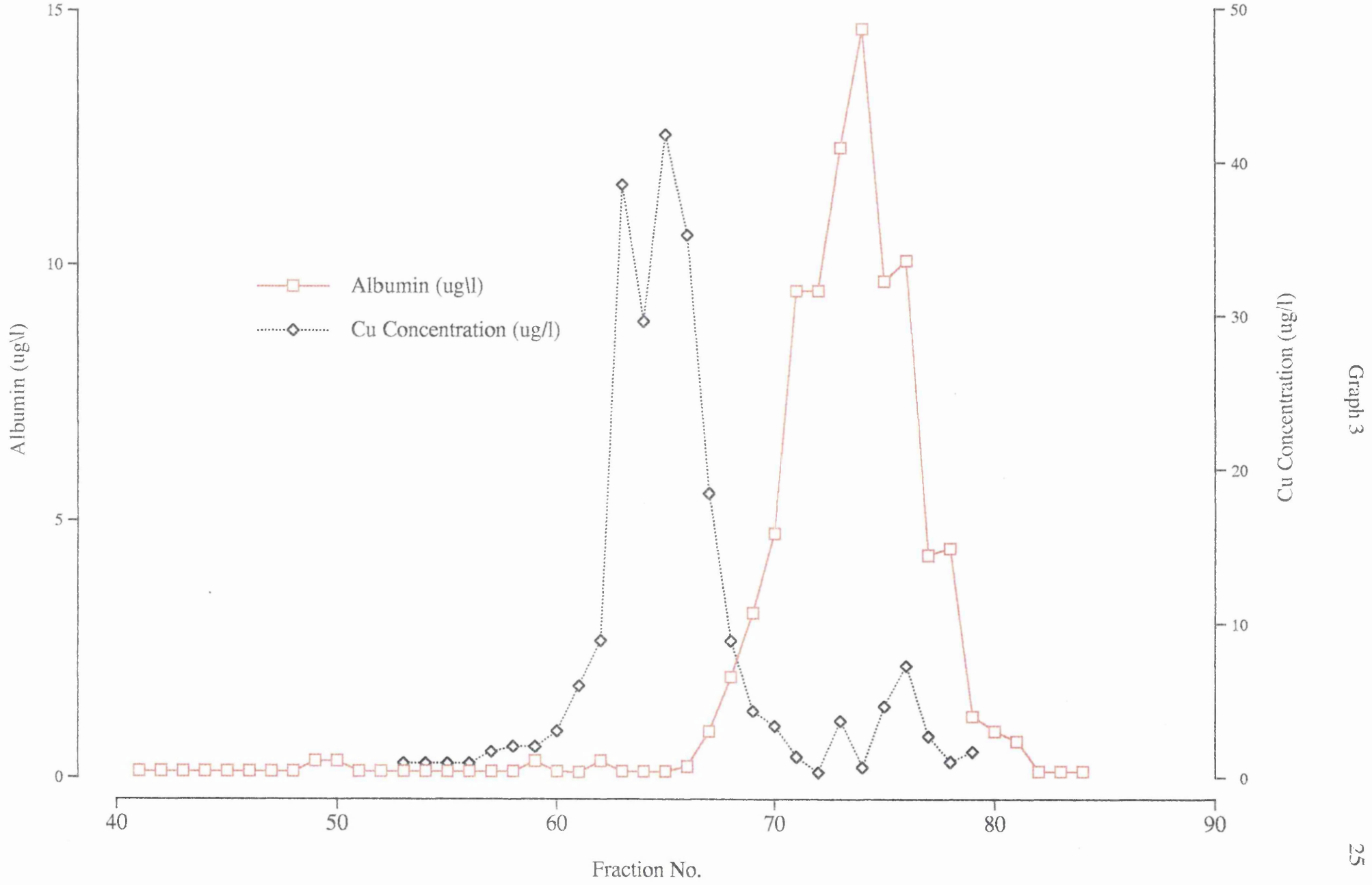
When the gel is packed into a 1m long column with 200mM Tris-HCl pH 7.4 as the liquid phase the conditions are optimal for a 1-2ml serum sample. A sample is run at 0.5ml/min for 14 hrs with the collection of one hundred 4ml fractions (Gardiner 1982). Protein concentration is measured as the absorbance of the solution at 280mm in a quartz cuvette (Graph 1). Copper is determined in each fraction by electrothermal (carbon furnace) atomic absorption spectrometry (see method 5) and albumin by immunoturbidity (Centrifichem, Atlantic Antibodies) (Graphs 2, 3). Attempts to measure caeruloplasmin by the same method were unsuccessful because large dilutions of the serum sample made the concentration of this protein undetectable by these means.


UV ABSORBANCE OF COLUMN FRACTIONS
1.5
1
0.5
0
U.V. Absorbance (280nm) 2AUFS
Ultra Violet Absorbance (280nm)
0
25
50
75
100
Fraction Number
Graph 1

ALBUMIN IN COLUMN FRACTIONS



Graph 2


COPPER VERSUS ALBUMIN
Albumin (ug\l)
Cu Concentration (ug/l)
Albumin (ug\l)
Cu Concentration (ug/l)
Fraction No.
0
5
10
15
0
10
20
30
40
50
40
50
60
70
80
90

Graph 3

RESULTS AND DISCUSSION

This method gives some information as to the distribution of Cu among the serum proteins. Copper is seen to be localised in two distinct fractions with a higher and lower copper content. These fractions can in turn be shown to cross-react with caeruloplasmin and albumin antibodies. This agrees with previous observations that up to 95% of serum copper is bound to caeruloplasmin and a smaller percentage, less than 5% to albumin. In gel filtration experiments it is generally assumed that complete separation occurs where the molecular weight of two proteins differ by a factor of two, and that one is about twice the size of the other. The molecular weights of Cp;132kDa and Alb; 67kDa fulfil this theoretical requirement but as graphs 2 and 3 show, the actual separation is not complete and takes considerable time, even for a small sample volume. The application of this system to the separation of albumin at an average of 40-80 g/l in serum and of Cp at an average 0.3 g/l is not feasible if we wish to quantify ^{65}Cu uptake by these proteins. The dilution factor of 1:400 makes it difficult to measure the concentration of caeruloplasmin by immunoturbidity.

ION EXCHANGE

Gel filtration cannot separate albumin from Cp with the necessary resolution. Caeruloplasmin because of its lability, needs to be separated rapidly to preserve its structural integrity (Dwulet 1981, Gutteridge 1985). The average concentration of Cp in human serum is 0.3g/l (Gibbs 1979, Louro 1989). To have enough Cp to measure at normal concentrations and those with lowered levels at least one to five mls of serum is required. The serum sample is stabilised with the protease inhibitor (ε–ACA) and diluted 1:1 with buffer (1 X PBS). The dilution reduces the viscosity of the sample and adjusts the ionic strength to a level suitable for ion exchange.

PROCEDURE

The basis for optimising any chromatographic scheme are considerations of speed, capacity, resolution and recovery. Media for ion exchange give high flow rates, high affinity protein binding and allow the application of a relatively large sample compared to the column volume. Ion exchange separates proteins according to differences in electrostatic charge and is highly selective. The charge of a protein is characterised by it's isoelectric point (pI) which is the pH at which it carries no net charge.

When at a pH below its pI the protein will carry a positive charge and at a pH above it's pI it will carry a negative charge. The pI determines whether a positively charged exchange media (an anion exchanger) or a negatively charged media (a cation exchanger) is used. The pH of the buffer determines the charge on the proteins and the extent to which they bind to or pass through the media. For proteins such as albumin and Cp with pI's of 4.9 and 4.4 respectively, a pH of at least one unit above the pI will ensure a sufficient charge on the proteins to allow a weak anion exchanger to be used.

SEPHAROSE

The ideal gel must have good stability to allow the high flow rates needed for rapid separations. Such a gel is DEAE-Sepharose Fast Flow (Pharmacia) based on agarose beads with 45-165μm diameter. The exchange group di-ethylaminoethyl (DEAE) is attached to a bead structure of 6% highly cross-linked agarose by stable ether linkages. The resulting structure is macroporous with good capacity for proteins of 1-1000 kDa, has excellent flow properties and low non-specific adsorption of proteins.

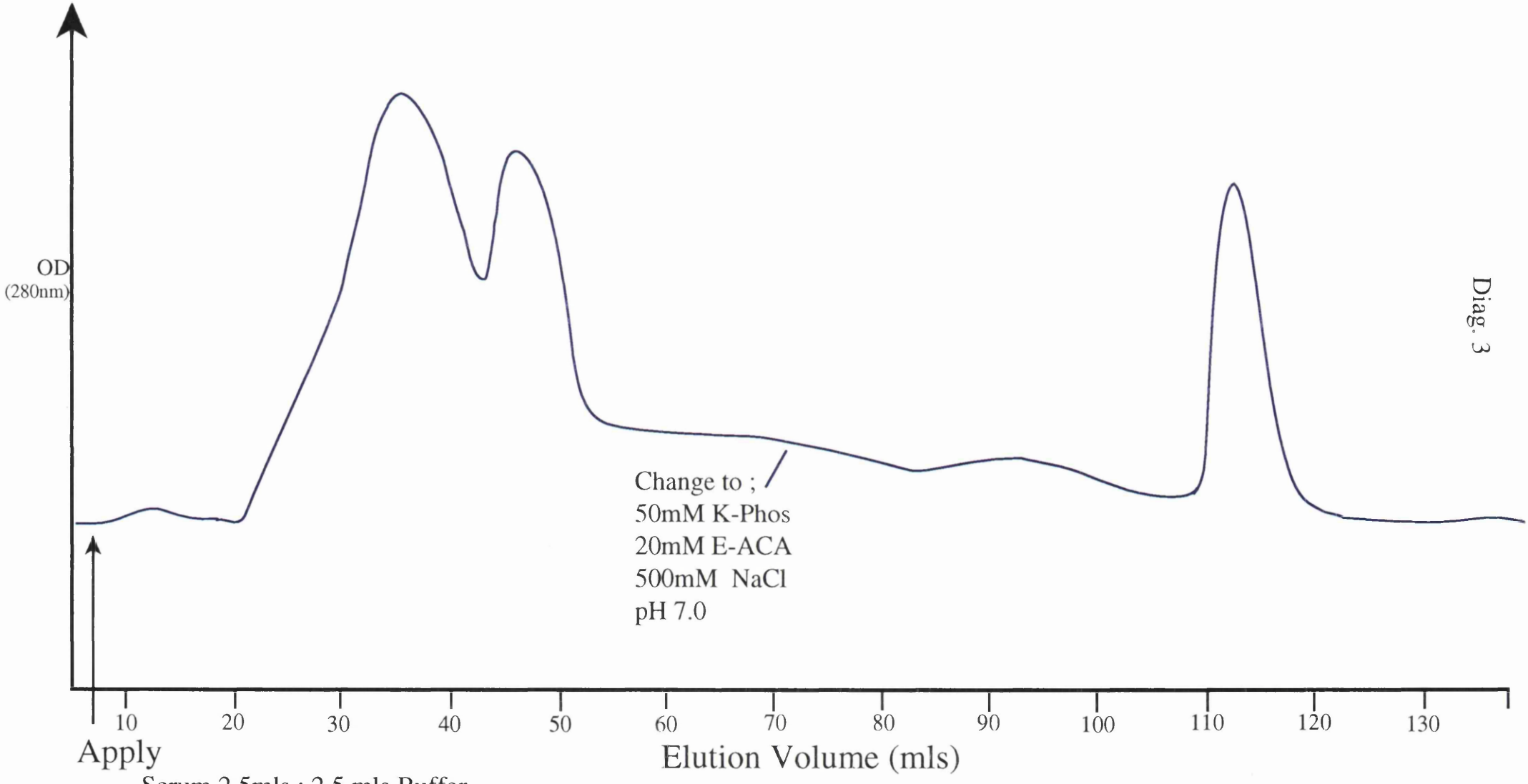
CONDITIONS

Ion exchange separates the components of samples carrying different net charges of varying strengths. Anion exchange uses a gel bearing a positive charge (DEAE) to which proteins carrying the opposite net charge to the gel bind by electrostatic forces. The charge of the gel is manipulated with the use of a counter ion, in this case, chloride (Cl^-). Proteins are then displaced and selectively eluted from the column with an increasing salt gradient. The greatest degree of fractionation is obtained when the protein of interest binds to the column and other molecules pass through. This involves the use of two buffers, the first a 'running' buffer and the second an 'elution' buffer. The running buffer (1xPBS + 20mM ε-ACA) carries the majority of the sample through the column unbound. The elution buffer (1xPBS, 0.5M NaCl + 20mM ε-ACA) charges the column with chloride counter-ions before equilibration with running buffer. Elution buffer is then used either by increasing the salt gradient to selectively remove more strongly attached proteins or in a single step to concentrate all proteins displaced by the NaCl content of the elution buffer. Stronger (2M) NaCl solutions are used to remove other ionically bound proteins and 1M NaOH to remove precipitated proteins and lipoproteins. To limit contamination a periodic, "trace metal" removal with 200mM EDTA, is part of the normal procedure (method 13). The diluted serum sample is applied at 1ml/min to a 1.7 x 20cm column packed with DEAE- Sepharose Fast Flow at room temperature to give the chromatogram (Diag3).

Start Buffer is:
PBS pH 7.2 / 50mM NaCl

FPLC CHROMATOGRAM

(DEAE Sepharose (FF))



Serum 2.5mls : 2.5 mls Buffer
1 ml / min, 1cm / ml, 2 AUFS

Diag. 3

The peak containing Cp is eluted in a single step in elution buffer in a volume of 10-15mls. After concentration (method 8) of the peak fractions, the absorbance at 280 and 610nm is used to gauge the purity of the separated Cp (method 2).

OTHER METHODS

In addition to ion exchange and gel filtration two other methods of isolating caeruloplasmin were investigated. These remove major serum proteins thereby simplifying the separation. The first involves the precipitation high MW proteins with polyethylene glycol (PEG). The second uses affinity chromatography to selectively retain albumin, and separate it from Cp.

PEG PRECIPITATION

Polyethylene glycol is a polymer that binds proteins having a suitable surface for adhesion. It also has the useful property of stabilising Cp in solution without the use of ε-ACA which is potentially toxic.

PEG, MW 4000, is made up in water or buffer as a 40% w/v solution. This was mixed 1:1 with serum samples to give a fraction containing Cp. After precipitation overnight in the cold room (4-10^{0}C) samples are centrifuged for 15mins at 3,000rpm in a benchtop centrifuge. The supernatant is then recovered for further purification.

The procedure is mild and does not affect enzymatic activity. It is a most useful step in the large scale isolation of undegraded Cp from "outdated" human serum (Oostuizen 1985).

AFFINITY CHROMATOGRAPHY FOR ALBUMIN

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly bound to a ligand attached to the gel matrix which has affinity for a group of proteins which share a common binding site. The ligand used is a dye, Cibacron Blue 3G-A, covalently attached to the highly cross linked agarose gel Sepharose CL-6B (Pharmacia). The dye binds a variety of proteins including coagulation factors, interferon and albumin. The mechanism of the molecular interaction is well documented (Gianazza 1982, Arnaud 1982). Some proteins interact biospecifically, such as albumin and interferon, whereas other proteins (such as Cp) bind in a less specific manner by electrostatic or hydrophobic attraction. The critical parameters of pH, ionic strength and temperature determine the extent of binding.

The separation of Alb from Cp is carried out at near the physiological pH and at room temperature. Serum samples (1-3mls) are diluted 1:1 with buffer (PBS pH 6.9) to reduce viscosity and adjust pH. The sample is then applied at 2mls/min to a 5ml 'HiTrap Blue' column (Pharmacia). Cp is bound to the column though not as strongly as Alb. Cp can be then eluted with PBS containing 650mM NaCl while albumin remains bound. Finally, albumin is desorbed with 0.5M NaCNS, but it's copper remains attached to the column.

The application of affinity chromatography with Blue Sepharose to the separation of Alb from Cp is feasible. However, it requires the use of three buffers which makes the processing of multiple serum samples time consuming and reproducible separations are difficult to achieve.

STABLE ISOTOPE STUDIES

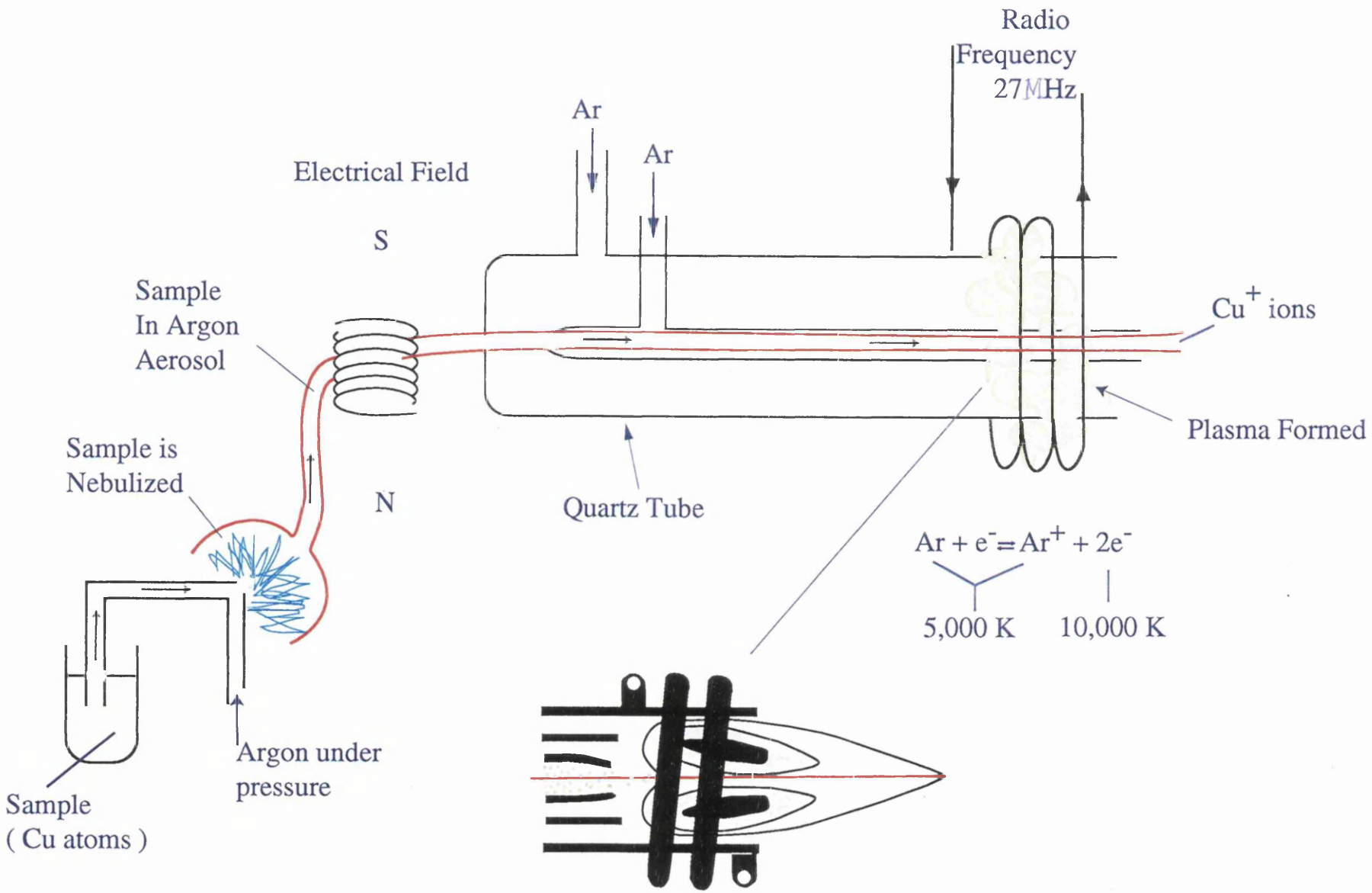
To study the kinetics of copper absorption, it's tissue distribution and excretion requires the use of oral and/or intravenously administered copper tracers. Radioactive tracers are relatively easy to measure but the half-lives of the copper isotopes are short and the radiation dosage unacceptable for pregnant women and for children. There are two copper radio-isotopes that can be used, ^{64}Cu (half-life 12.9hrs) and ^{67}Cu (half-life 61.9hrs). They do not allow long term metabolic studies. This has resulted in good definition of copper distribution in the first few days and hours following administration but not during the following weeks.

Copper has two stable isotopes ^{63}Cu and ^{65}Cu with natural abundance's of 69.09% and 30.91% respectively. The use of stable isotopes depends on an accurate measurement of the ^{65}Cu : ^{63}Cu ratio in serum, now possible by ICP-MS (Lyon 1990). The proportion of ^{65}Cu in serum is increased by giving ^{65}Cu orally. Dosages for adults of 3mg, irrespective of body size are given. This produces an *in vivo* enrichment of the less abundant ^{65}Cu in serum samples taken at various times after the dose. The enrichments can then be followed until the absorbed ^{65}Cu disappears from the circulating blood serum and is excreted. The pattern of ^{65}Cu serum enrichment over days or weeks can be used to examine copper handling in both health and disease.

ICP-MS EQUIPMENT

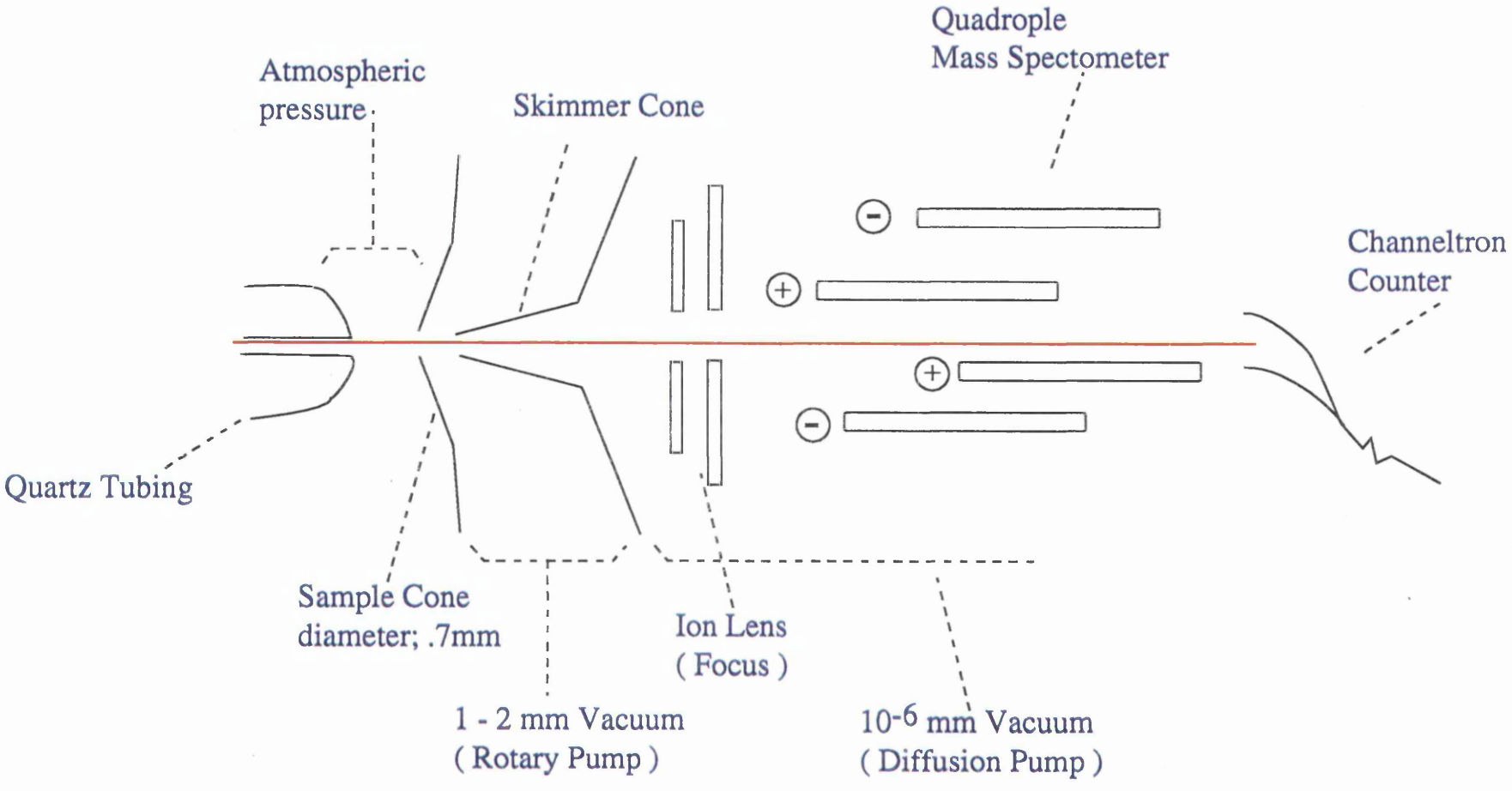
The machine used was a Plasmaquad PQ2+ ICP-MS (VG-Elemental: Fisons). The ions for mass analysis are produced conveniently from liquid samples by the inductively coupled argon plasma (ICP) (Diag.4). The singly charged Cu^+ analyte ions generated in the ICP are extracted into and /

ICP IONIZATION



and/ measured with a quadropole mass spectrometer (Diag.5). The liquid sample is pumped along plastic tubing by a peristaltic pump to the nebulizer. This converts the liquid sample into an aerosol before introduction into the ICP spray chamber. The nebuliser used was a "Mienhard" concentric nebuliser which exhibits good long term stability, but because of the very small annular gap is prone to blockage by particles in the analyte solution and by solutions having high solute concentrations. The sample is thereby contained in a laminar flow of argon which is introduced to the ICP torch. A "Fassel" torch is used, consisting of three concentric silica tubes with tangential gas entries to the outer and intermediate tubes. Aerosol is introduced through the small-diameter tube along the torch axis. This "injector" inner tube has a constricted exit at a distance from the end of the outer tube and coinciding with end of the intermediate tube. The end of the outer tube is encircled by three turns of copper coil through which a high current generated by a crystal-controlled radio frequency of 27.1MHz is passed to form the plasma. The plasma is an ionised gas with high excitation, ionisation and electron temperatures. The high temperature region of the plasma exists as $Ar + e^- \leftrightarrow Ar^+ + 2e^-$ providing metastable (Ar^M) and charged (Ar^+) ions by "Penning ionization" and "Charge transfer" respectively. It is self-sustaining after initial seeding with a few electrons from a Tesla coil. The sample in its carrier gas stream is injected into the high temperature atmospheric pressure argon plasma where it is desolvated, atomised (excited) and ionised. The central portion of the annular plasma is extracted into a low pressure region (10^{-2} Torr, maintained by rotary vacuum) through the sample cone. The plasma material is transported as a supersonic jet, a small proportion (1-3%) of which passes through the skimmer cone orifice. Positive ions are extracted and focused by an ion lens system into the quadropole mass filter and filtered according to their mass to charge ratio. Individual ions are detected by an ion counting electron multiplier known as a "channeltron" counter.

ION FOCUSING



The channeltron can operate at high beam currents in analogue mode. The horn shaped channel of the counter is made of made of glass with a resistive surface. This device can be thought of as a physical charge amplifier. Impacting ions release secondary electrons. These undergo further collisions with the tube walls, releasing more electrons, and an exponential avalanche builds up down the channel giving a gain of around 10^8.

SAMPLE REQUIREMENTS

The direct measurement of the $^{65}Cu/^{63}Cu$ ratio is not possible in whole serum because of polyatomic interference. Sodium and argon ions combine to give a product $Na\text{-}Ar^+$ with the same mass as ^{63}Cu. The observed ratio is not accurate and the serum sample must be desalted to separate Cu from Na (method 1). The protein moieties also introduce polyatomic interference, from sulphur $^{32}S\text{-}^{33}S$. Addition of concentrated nitric acid (20µl per ml (2%)) ensures that proteins are precipitated and sulphur removed while the Cu stays in solution. Samples were then made up to 10mls in deionised water and the Cu concentration determined by flame AAS (method 6). Subsequent dilution with 2% nitric acid to < 0.3µmol/Cu/L ensured that the channeltron operated only in pulse counting mode. Too high a Cu concentration results in the channeltron switching to analogue mode which is not desirable during pulse counting.

Samples that were stored in the cold room required centrifugation (15mins at 3,000rpm) immediately before analysis to remove any precipitate or particulate matter.

RUN CONDITIONS

Samples were presented to the instrument in a volume of 10mls and five replicate measurements of the $^{65/63}$Cu ratio made over five minutes (method 11). A blank and five standards were run before the samples to ensure consistent measurement at the start of the run and give the baseline for mass bias correction. A standard was included after every five samples for mass bias correction. Printouts give actual counts (blank corrected) for ^{63}Cu and ^{65}Cu ions for the five readings with a mean and standard deviation (%SD). An acceptable imprecision is < 0.5%.

MASS BIAS CORRECTION

The blank corrected $^{65/63}$Cu ratio produced by the ICP-MS requires correction for mass bias. This is the tendency of the instrument to overmeasure or undermeasure the known ratio of the standard solution, determined by the measurement of the five initial standards. As the ^{65}Cu atom is heavier than ^{63}Cu, it is subjected to stronger forces on passing through the quadropole focusing field. The natural ratio is obscured by this artefact. Correction is made to the blank corrected ratio (automatically calculated) by multiplication with a discrimination factor (f). This factor is worked out by taking the natural $^{65/63}$Cu ratio (0.4474) and dividing it by the mean ratio of the five standards. The amount of mass bias occurring between control points is added or subtracted in a linear fashion according to the direction of the bias. The 'f' factor is then used to multiply the blank corrected ^{65}Cu/^{63}Cu ratio obtained to give the true ratio. This may occur for any number of reasons, such as time of day (other instruments being turned on and off) or duration of operation. Although the instrument may not measure the same ratio on a sample with a known $^{65/63}$Cu ratio the control samples allow for correction of any difference.

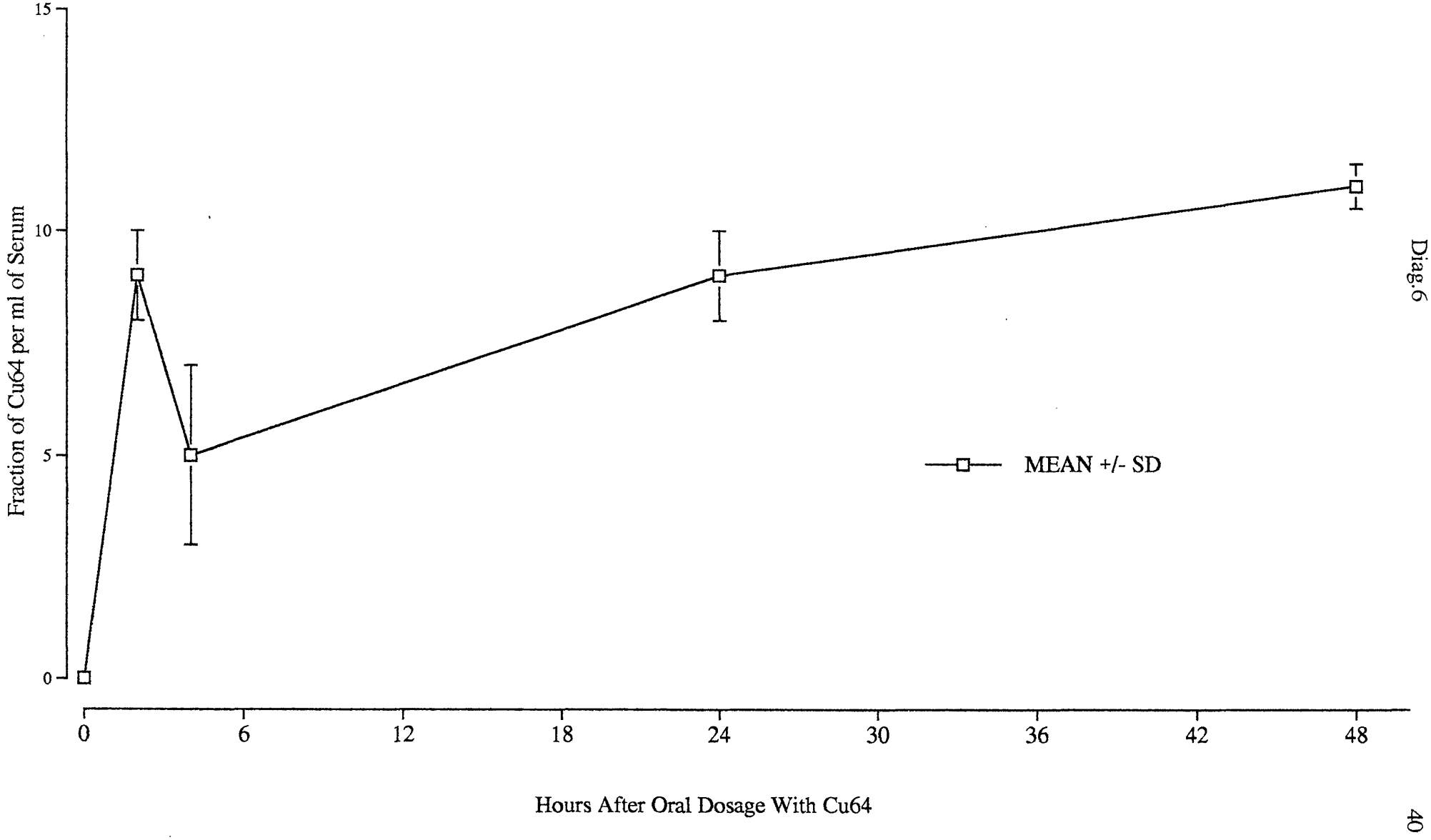
The percent enrichment (En%) is worked out by subtraction of the natural ratio from the measured ratio as a percentage of the natural ratio by the following formula;

$$\%En = \frac{65Cu/63 \text{ corrected} - Cu65/63 \text{ natural}}{65Cu/63 \text{ natural}} \times 100$$

^{64}Cu RESULTS

The results of radio active studies performed in the 1960's (Diag.6 after Sternlieb) first elucidated the picture of copper turnover. It was seen that one or two hours after ingestion radio active Cu appears in the circulating blood plasma as a sharp peak over 1-2 hours subsiding to a trough at about six hours. There is then a gradual reappearance of radioactivity (cpm) reaching a maximum after seventy-two hours. The initial peak is produced as some of the copper on albumin transported from the gut via the hepatic portal vein bypasses the liver at the first pass and enters the peripheral blood. The secondary rise occurs after copper incorporation into caeruloplasmin. Taking the ratio for the secondary rise to the initial peak should give a value greater than one for normals and less than one for failure to incorporate into Cp and retention by the liver. The ratio of the initial peak to the trough was used to show the clearance and reappearance effect absent in individuals with disorders of Cu incorporation into Cp.

Cu64 IN 19 CONTROL SUBJECTS

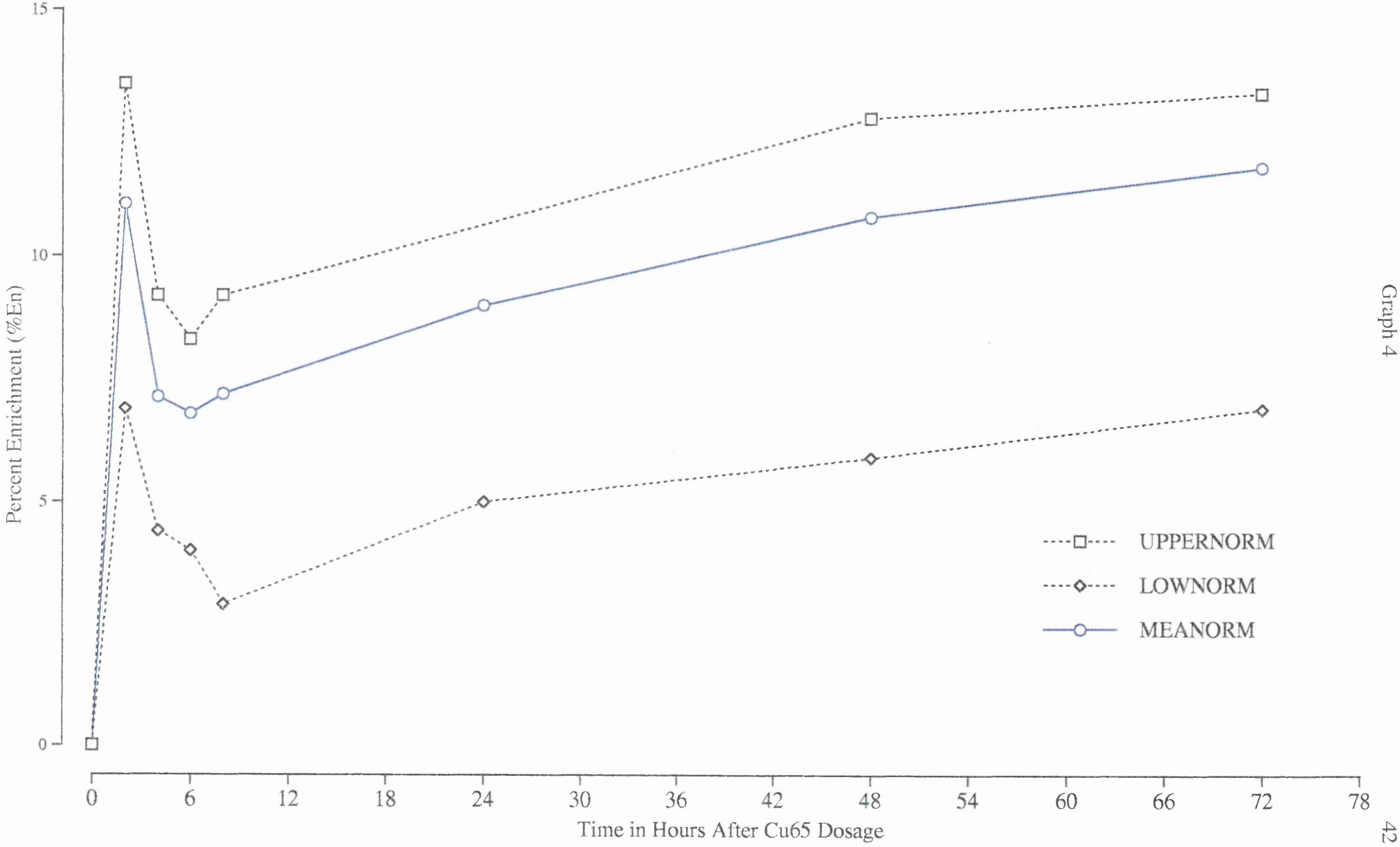


Diag. 6

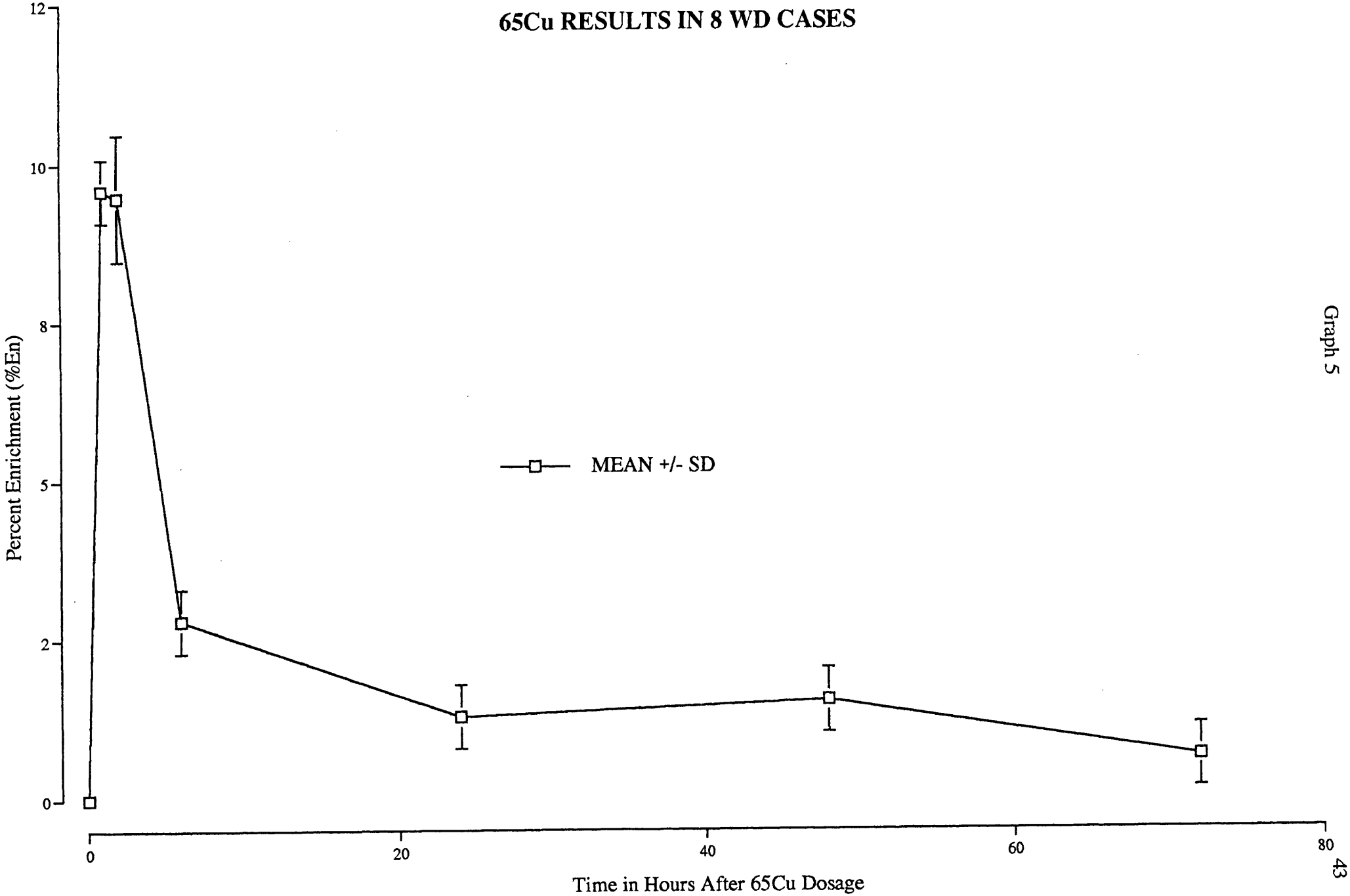
Stable isotope investigations have now been carried out on fifteen normals (Graph 4) and eight WD patients (Graph 5). The shape of the graphs are similar to those obtained by radioactive copper. The initial rise at 1-2hrs is present in both graphs but the secondary rise, as ^{65}Cu appears on caeruloplasmin, is shown by normal subjects and is absent in WD patients. When expressed as ratios, the 72/2 hour ratio is greater than 0.5 in normals and less than 0.5 in affected individuals. The 72/6 hour ratio is greater than 1.5 in normals and considerably less in WD cases (0.15+/-0.06 n=7). These ratios are of diagnostic importance in equivocal cases (Lyon 1995).

The stability of the isotope offers other possibilities, in that the ability to follow ^{65}Cu over a longer course of time, allows the estimation of the biological half-time ($t_{1/2}$) of copper in serum. The four subjects studied; L.W., B.S., S.M. and P.G., have half-times of 27, 27, 17 and 21 days respectively, with an average of 23 days.

65Cu ENRICHMENTS IN 15 NORMAL SUBJECTS



Graph 4


65Cu RESULTS IN 8 WD CASES
12
10
8
5
2
0
Percent Enrichment (%En)
MEAN +/- SD
0
20
40
60
80
Time in Hours After 65Cu Dosage
Graph 5

THE METABOLIC STUDY

A study of copper metabolism using the stable isotope was designed (method 14), which involved measurement of ^{65}Cu enrichment in whole serum, caeruloplasmin, urine and faeces. The biological half-time of copper can be calculated, and the ^{65}Cu enrichment of faecal excreta allows calculation of Cu retention (Diags 7, 8, 9, 10). For this calculation it is important that all the plastic pellets given as markers are recovered. The pellets are made of a radio-opaque material and can be counted in the faecal samples by X-ray. Five consecutive collections are made on the five days after the ^{65}Cu dose. The retention of the given dose in four normal subjects after five days was from 42.2 - 54.6%. This indicates that over the given time, around half the ^{65}Cu is excreted by the normal subject.

Four volunteers, two male and two female provided an average of fifteen blood samples of 10mls each at frequent intervals over five weeks (Diags 11,12). The serum was split into portions, 2.5mls for total serum Cu and the remainder (usually 1-3mls) for Cp isolation. Samples for total serum ^{65}Cu were prepared for ICP-MS by gel filtration (method 1). Cp isolation was performed as follows:

				COPPER-65 METABOLIC STUDY-FAECAL SAMPLES							
							L.W.				
Code	Pellets	Total Dry Wt.	(65/63)m	(65/63)0	Sample Wt.	Vol.	Conc.	Faecal Cu conc.	Total Cu	Cu-65 tracer	Cu-65 tracer
		g			g	ml	µmol/l	µmol/g(dry)	µmol	µmol	mg
L0	0	43.1	0.4474	0.4474	0.1108	25	3.20	0.7220	31.1191	0.0000	0.0000
L1	11	41.5	1.1622	0.4474	0.1225	25	4.40	0.8980	37.2653	12.3754	0.7865
L2	5	15.8	0.9996	0.4474	0.0650	25	2.90	1.1154	17.6231	4.8888	0.3107
L3	4	29.9	0.6418	0.4474	0.1227	25	4.00	0.8150	24.3684	2.8985	0.1842
L4	0	37.7	0.5195	0.4474	0.1387	25	3.30	0.5948	22.4243	1.0689	0.0679
	20	out of 20 recovered						TOTAL EXCRETED		21.2316	1.3493
								5 DAY RETENTION (%)		**54.6**	

Key

m = measured ratio

0 = natural ratio

COPPER-65 METABOLIC STUDY-FAECAL SAMPLES

B.S.

Code	Pellets	Total Dry Wt. g	(65/63)m	(65/63)0	Sample Wt. g	Vol. ml	Conc. µmol/l	Faecal Cu conc. µmol/g(dry)	Total Cu µmol	Cu-65 tracer µmol	Cu-65 tracer mg
B0	0	34.0	0.4474	0.4474	0.0483	25	1.20	0.6211	21.1180	0.0000	0.0000
B1	9	27.8	1.5959	0.4474	0.0497	25	2.00	1.0060	27.9678	12.4299	0.7899
B2	8	23.9	1.2600	0.4474	0.1146	25	4.40	0.9599	22.9407	8.2859	0.5266
B3	2	23.3	0.7295	0.4474	0.0696	25	2.80	1.0057	23.4339	3.8397	0.2440
B4	1	13.9	0.5410	0.4474	0.1039	25	3.30	0.7940	11.0371	0.6734	0.0428
	20	out of 20 recovered						TOTAL EXCRETED		25.2290	1.6033
								5 DAY RETENTION (%)		**46.1**	

Key

m = measured ratio

0 = natural ratio

COPPER-65 METABOLIC STUDY-FAECAL SAMPLES

S.M.

Code	Pellets	Total Dry Wt. g	(65/63)m	(65/63)0	Sample Wt. g	Vol. ml	Conc. µmol/l	Faecal Cu conc. µmol/g(dry)	Total Cu µmol	Cu-65 tracer µmol	Cu-65 tracer mg
S0	0	6.99	0.4474	0.4474	0.0928	25	3.60	0.9698	6.7791	0.0000	0.0000
S1	18	75.7	0.8509	0.4474	0.0895	25	3.00	0.8380	63.4358	13.8919	0.8828
S2	2	35.9	0.8472	0.4474	0.1211	25	4.00	0.8258	29.6449	6.4453	0.4096
S3	0	50.1	0.5212	0.4474	0.1041	25	2.70	0.6484	32.4856	1.5832	0.1006
S4	0	46.16	0.4855	0.4474	0.1300	25	3.90	0.7500	34.6200	0.8920	0.0567
	20	out of 20 recovered						TOTAL EXCRETED		22.8124	1.4497
								5 DAY RETENTION (%)		**51.2**	

Key

m = measured ratio

0 = natural ratio

COPPER-65 METABOLIC STUDY-FAECAL SAMPLES

P.G.

Code	Pellets	Total Dry Wt. g	(65/63)m	(65/63)0	Sample Wt. g	Vol. ml	Conc. µmol/l	Faecal Cu conc. µmol/g(dry)	Total Cu µmol	Cu-65 tracer µmol	Cu-65 tracer mg
PG0	0	34.6	0.4474	0.4474	0.1195	25	3.40	0.7113	24.6109	0.0000	0.0000
PG1	5	45.2	0.6492	0.4474	0.0989	25	3.40	0.8595	38.8473	4.7750	0.3035
PG2	3	11.8	1.1143	0.4474	0.0959	25	4.00	1.0428	12.3045	3.8987	0.2478
PG3	7	24.5	1.0700	0.4474	0.0844	25	4.30	1.2737	31.2056	9.4284	0.5992
PG4	5	128.3	0.5810	0.4474	0.0639	25	2.10	0.8216	105.4108	8.9480	0.5686
	20	out of 20 recovered						TOTAL EXCRETED		27.0502	1.7190
								5 DAY RETENTION (%)		**42.2**	

Key

m = measured ratio

0 = natural ratio

FPLC Isolation Procedure

1) 1-3mls serum + 20mM ε-ACA (protease inhibitor).
2) Dilute 1:1 with start buffer to reduce viscosity and ionic strength.
3) Filter through 0.45μm filter to remove particulate matter.
4) Bind Cp to DEAE-Sepharose FF allowing most other proteins to pass through
   The column is run at 2ml/min with 1 X PBS pH 6.8 as the buffer.
5) Elute the bound Cp stepwise or with a salt gradient increasing to
   650mM NaCl concentration.
6) Read absorbance of eluted fractions at 280 and 610nm (method 2).
7) Pool, concentrate and desalt fractions with 610/280nm absorbance ratios > 0.02.
8) Sample further purified on Mono Q column or by gel filtration on Superdex 200HR
9) Cp peak collected and measured by immunodiffusion (Behring).
10) Fast desalting and buffer removal on Sephadex G25 (superfine).
11) Precipitation of proteins with 100μl/ml conc. nitric acid.
12) Centrifuge at 3,500rpm for 15mins.
13) Make up to 10mls with deionised water (sample for ICP-MS).
14) Measure copper concentration on GBC-Flame AA (method 6).

Care must be taken at each stage to minimise sample losses on filters and containers, limit the introduction of air bubbles into columns and prevent contamination. The procedure takes time in particular the initial separation on DEAE sepharose which improves with slower runs and larger samples. The average is 45mins per sample. A number of runs may be completed before it is

necessary to clean the column, by washing with NaCl, NaOH and EDTA successively (method 13).

A freshly poured column was used for each volunteer. The elution of Cp with NaCl was found to be effective at a minimal concentration of 650mM. This is still high enough to induce a rapid breakdown of the molecule after elution so it is important to remove the salt before storage (method 8).

COPPER RECOVERY

Copper recovery is a measure of the efficiency of the separation since 95% of Cu is Cp bound. An initial total serum Cu measurement was made on a fresh serum sample by the routine analytical procedure which had satisfactory bias and imprecision in national QC schemes.

The results were as follows;

Volunteer	Serum Cu (μmol/L)	Reference Interval
L.W. (Male)	15.0	12-24μmol/L
B.S. (Female)	17.5	
S.M.(Female)	26.5	
P.G. (Male)	12.5	

Fractions eluted during sample runs not being used in subsequent separations were measured to ensure that they contained no more than 0.1μmol/L Cu before disposal. Fractions eluted during column cleaning with 200mM EDTA (a strong Cu chelator) were measured to account for Cu removed from albumin during chromatography on DEAE Sepharose. The copper in the samples prepared for ICP-MS was measured by flame AAS (Diag. 11).

COPPER IN ICP-MS PREPARATIONS

(μmol/Cu/L)

	L.W.	B.S.	S.M.	P.G.
T=0hrs	1.3	2.6	4.4	1.3
T=0.5hrs	2.6	4.3	7.5	0.6
T=1.5hrs	2.8	4.8	4.1	0.1
T=2hrs	1.8	5.1	2.1	0.1
T=2.5hrs	1.3	5.5	5.4	1.7
T=6.5hrs	2.2	5.9	6.4	1.9
T=24hrs	2.5	4.2	5.2	0.1
T=48hrs	0.1	4.2	4.3	1.6
T=72hrs	2.5	6.4	4.1	2.2
T=96hrs	2.3	2.6	6.9	1.9
T=1wk	3.5	3.1	9.8	1.5
T=2wk	2.6	3.6	4.9	2.6
T=3wk	3.5	3.7	3.7	1.2
T=4wk	2.2	4.3	2.9	0.8
T=5wk				0.6

An estimate of copper recovery from the FPLC column was made on the first serum sample from each subject.

Recovery was calculated as;

$$\frac{\text{Cu concentration in pooled Cp fractions (µmol/l)} \times \text{Volume of pool (l)}}{\text{Serum Cu concentration (µmol/l)} \times \text{Volume of serum used (l)}}$$

Recoveries from the initial serum samples were as follows;

L.W.	74.3%
B.S.	81.9%
S.M.	81.8%
P.G.	50.4%

CAERULOPLASMIN RECOVERY

Initial Cp values were obtained by nephelometry because of the QC (see p.50) on this measurement. The following values were obtained;

Volunteer	Value	Reference Interval
L.W.	254mg/l	200-500mg/l
B.S.	280mg/l	
S.M.	474mg/l	
P.G.	194mg/l	

Caeruloplasmin was also measured by a single radial immunodiffusion (Behring) of 5µl from the concentrated product before desalting and buffer removal. The purity of the preparation was

gauged by spectrometry. Caeruloplasmin purity and recovery was calculated as shown in the following example;

L.W. - 254mg/l = 0.254 mg/ml x 2mls (volume of serum sample) = 0.509mg

- 0.509mg Cp present in initial sample.

Immunodiffusion plate results;	610/280nm Absorbance ratio;
1. T=0hrs -	0.033
2. T=0.5h 592mg/l	0.025
3. T=1.5 613mg/l	0.027
4. T=2 254mg/l	0.060
5. T=2.5 350mg/l	0.040
6. T=6.5 552mg/l	0.064
7. T=24 720mg/l	0.025
8. T=48 317mg/l	0.024
9. T=72 384mg/l	0.017
10. T=96 333mg/l	0.034
11. T1WK 592mg/l	0.039
12. T2WK 419mg/l	0.031

Total = 5136mg/l / 12 = 428mg/l

Average percentage recovery ; 428mg/l = 0.428mg/ml x 1ml (volume of conc.. fractions) = 0.428 mg

- 0.428/0.509 x 100 = 84.1%

Results

L.W. 84.1%

B.S. >90%) Beyond limits of

S.M. >90%) immunodiffusion plate. (Later found to be out of date)

P.G. 27.3% (This was due to overwashing the column with EDTA.)

ICP-MS RESULTS

The Cp isolated from the four subjects, was prepared for measurement of isotopic enrichment by ICP-MS (Diag.12). Results are presented as graphs on the following pages. The %En values are considerably lower for Cp than expected due to loss of copper during chromatography. This was investigated as follows:

CAERULOPLASMIN INCUBATION WITH ^{65}CU

It has been shown that it is possible to label caeruloplasmin with radioactive copper by exchanging a portion of the copper atoms *in vitro* under reducing conditions (Scheinberg 1957, Sternlieb 1961). The amount of copper incorporated is reported as 35-50% of total Cp copper. The exchange involves the shuttling of the copper atom between monovalent and divalent states under reducing conditions.

I found that the incorporation *in vivo* of ^{65}Cu into caeruloplasmin was at a lower level than expected. If this was due to an experimental artefact then solutions of Cp which had exchanged a portion of the natural copper for ^{65}Cu should show the difference before and after passing through the separation procedure.

^{65}Cu PERCENTAGE ENRICHMENT IN FOUR METABOLIC SUBJECTS

Time hrs.	L.W. Tot	L.W. Cp	B.S. Tot.	B.S. Cp	S.M. Tot.	S.M. Cp	P.G.Tot.	P.G. Cp
0	0	0	0	0	0	0	0	0
0.5	7.69	0	6.73	0	1.18	0	3.57	0
1.5	11.42	0	6.79	0.78	7.24	0	11.66	0
2	10.37	0.07	5.52	0.96	7.26	1.62	8.14	0
2.5	8.76	1.22	4.36	1.16	7.55	2.01	6.5	0.17
6.5	4.83	1.45	3.22	-	6.93	3.1	2.32	-
24	5.96	2.63	4.4	1.74	11.42	6.34	3.78	0.68
48	7.19	-	5.92	2.08	13.52	7.41	5.25	3.22
72	7.11	3.98	6.84	2.32	13.75	8.36	5.39	4.01
96	7.55	4.38	7.48	4.27	14.26	9.65	6.13	4.16
1wk:144	7.35	6.12	7.73	5.6	12.62	9.4	7.59	4.43
2wk:168	7.19	3.88	7.77	5.54	10.23	8.18	6.53	5.14
3wk:336	6.08	-	7.73	5.45	7.8	4.12	4.8	3.75
4wk:504	5.49	2.87	-	-	5.81	0.9	4.12	2.21
5wk:672	-	-	4.74	-	-	-	-	1.54

Key;

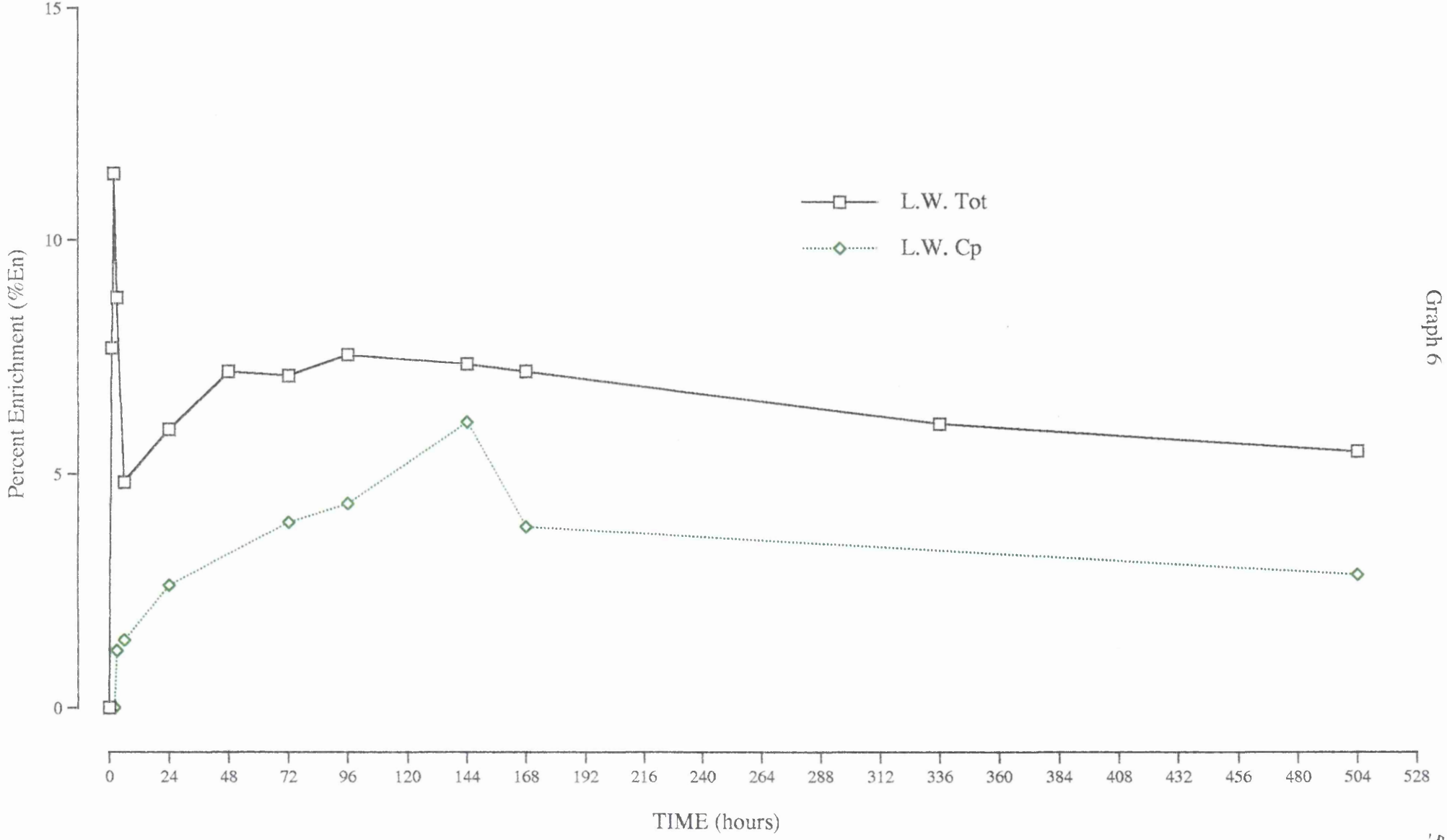
Tot. = Percent Enrichment in whole serum.

Cp. = Percent Enrichment on isolated caeruloplasmin.

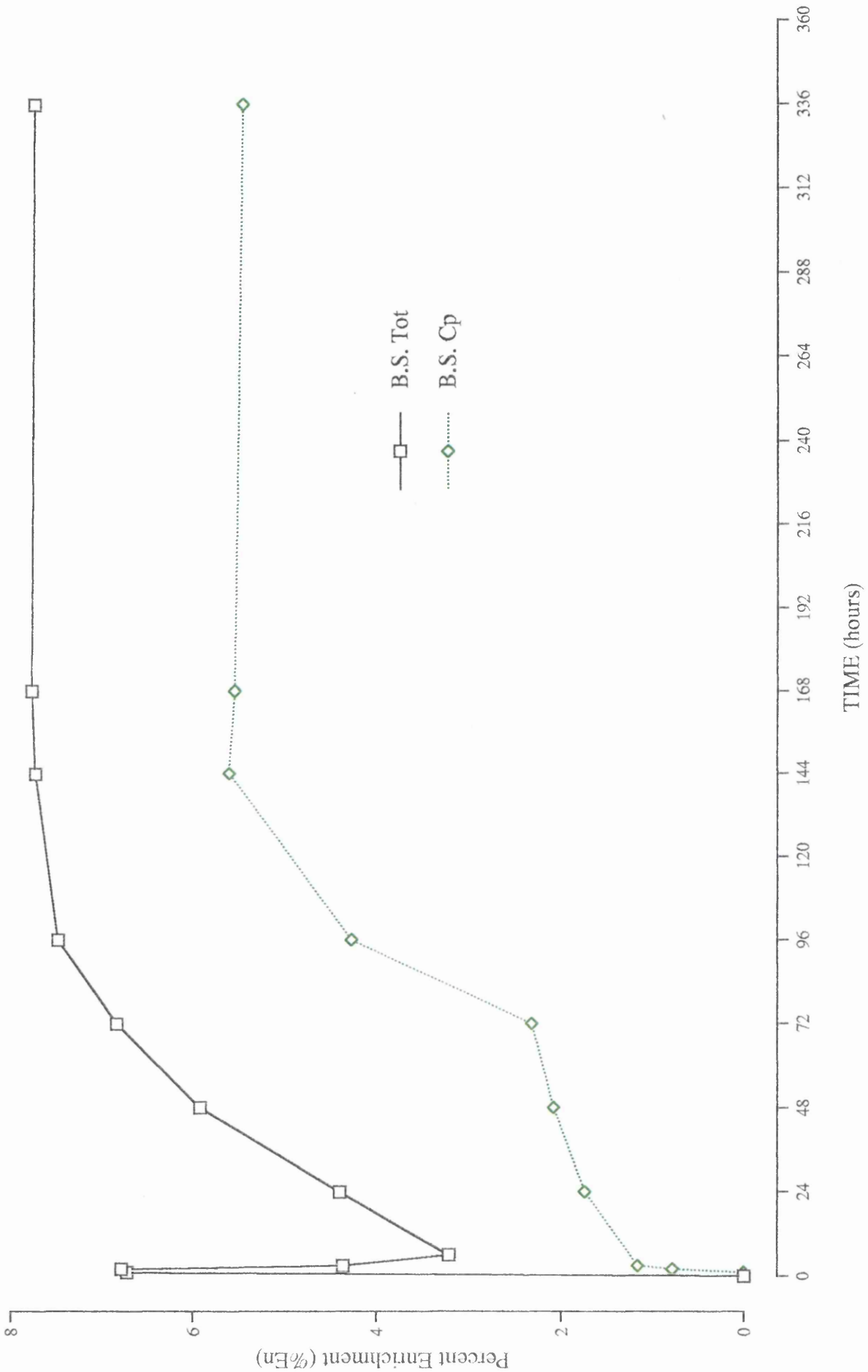
0 = Measured as 0.

- = Sample not collected or lost in process of separation.

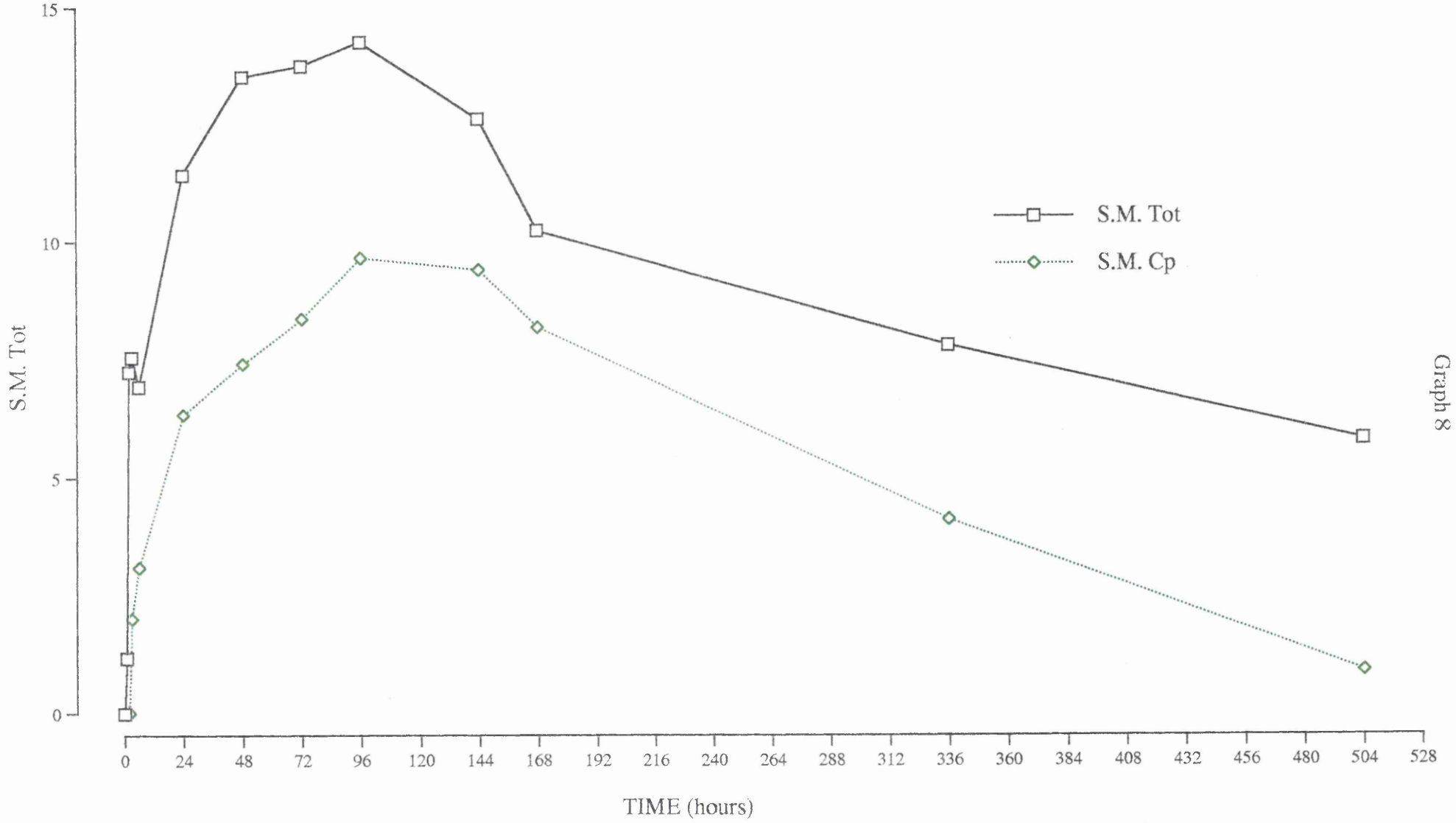
SUBJECT 1 'L.W.'



Graph 6

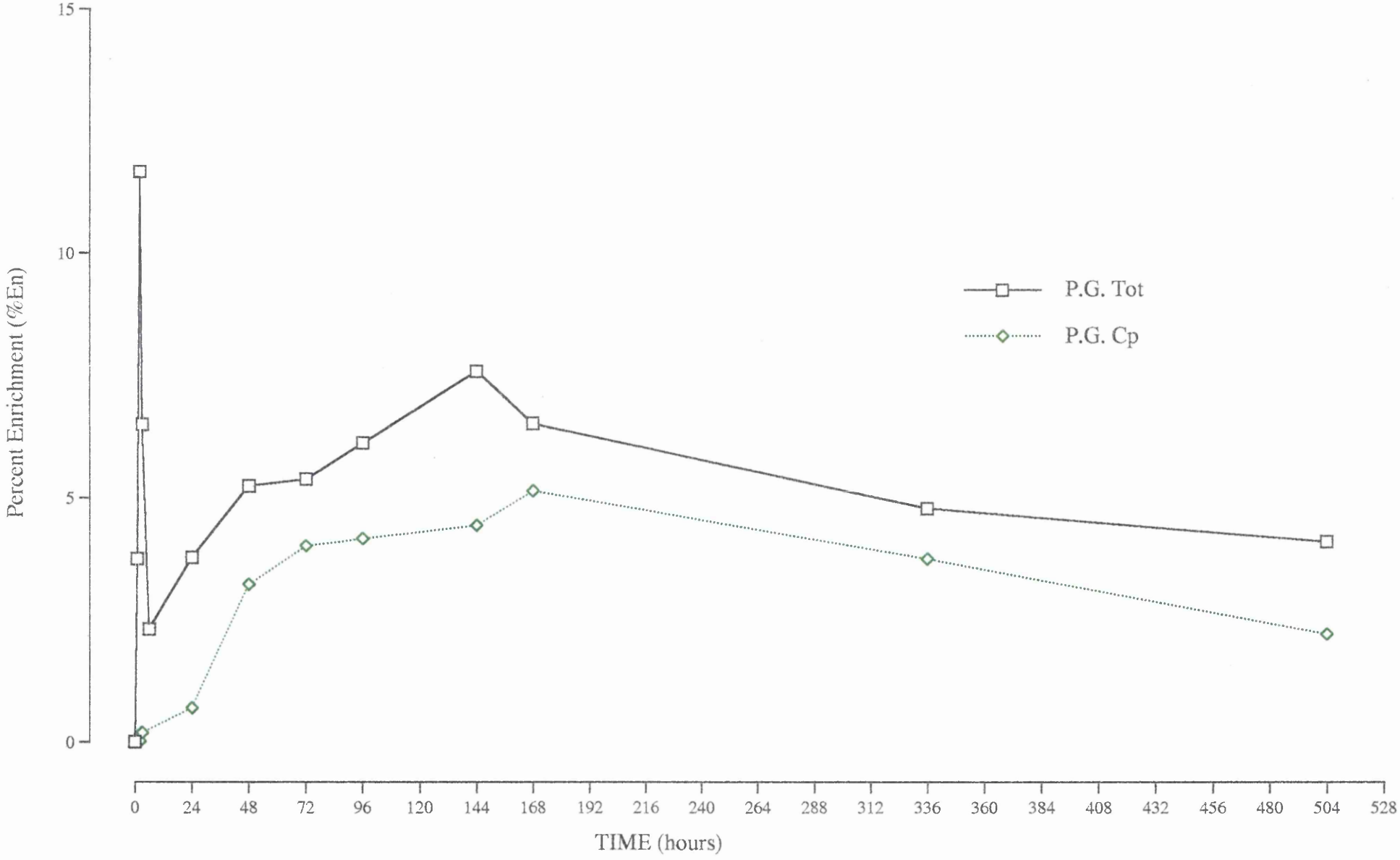

SUBJECT 2 'B.S.'
B.S. Tot
B.S. Cp
Percent Enrichment (%En)
0
2
4
6
8
TIME (hours)
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360

SUBJECT 3 'S.M.'



Graph 8

SUBJECT 4 'P.G.'



Graph 9

Experimental conditions

1) Materials

- 65mmol/L $^{65}Cu^{++}$ nitrate in nitric acid
- 100mM sodium acetate buffer pH 5.5
- Saline 0.9%
- 1M L-ascorbic acid
- Purified caeruloplasmin 1mg in 22µls

2) Volumes

- 2.5mls for PD-10
- 10mls for ICP-MS

3) Conditions

The success of the label is critically dependant upon time, temperature, concentration of substrate and reductant added, pH and ionic composition of labelling mix.

- Temperature 37^{0}C
- Time 1.5hrs under vacuum
- pH = 5.4
- Caeruloplasmin 0.3mg/ml
- 25mM L-ascorbic acid
- 20mM ε–amino caproic acid

Both commercial (Sigma) and FPLC purified Cp were incubated under the conditions described. Chelex 100 was used to remove loosely bound Cu (method 9) from all samples. Half were then

directly prepared for ICP-MS while the others were put through the FPLC procedure before being prepared for ICP-MS measurement. A trial incubation was carried out without vacuum and demonstrated the feasibility of labelling. Incubations were then performed for commercial and FPLC- isolated Cp.

Samples were prepared in triplicate with controls for both copper and caeruloplasmin. The experiment was conducted under vacuum with ascorbic acid as the sole reducing agent. The enrichment of caeruloplasmin with ^{65}Cu was 135% on average after removal of the ascorbic acid and excess ionic copper. After subjecting the caeruloplasmin sample to FPLC chromatography the enrichment fell to 94%En representing a 70% recovery of immunologically active protein. Since only caeruloplasmin was applied to the separation procedure the copper loss can be directly attributed to the chromatographic process. Ionic copper can therefore be exchanged with active caeruloplasmin to a significant extent.

Commercial Cp Results

	% Enrichment (see p.39)
1) Reaction buffer + caeruloplasmin	0.04
2) " + ^{65}Cu	0.00
3) Buffer, ascorbic acid , ^{65}Cu and Cp	134.43
4) "	135.12
5) "	135.62
6) Buffer, ascorbic acid, ^{65}Cu and Cp post FPLC	88.98
7) "	98.16
8) "	94.69

FPLC Isolated Caeruloplasmin

Caeruloplasmin was isolated as described from standard male serum (method 16) to give the following percent enrichments after incubation.

	Cu (μmol/L)	%En	Procedure
1)	5.9	204	PD-10 into water
2)	5.3	205	"
3)	6.8	208	"
4)	5.0	134	post-FPLC
5)	6.4	145	"
6)	7.4	135	"

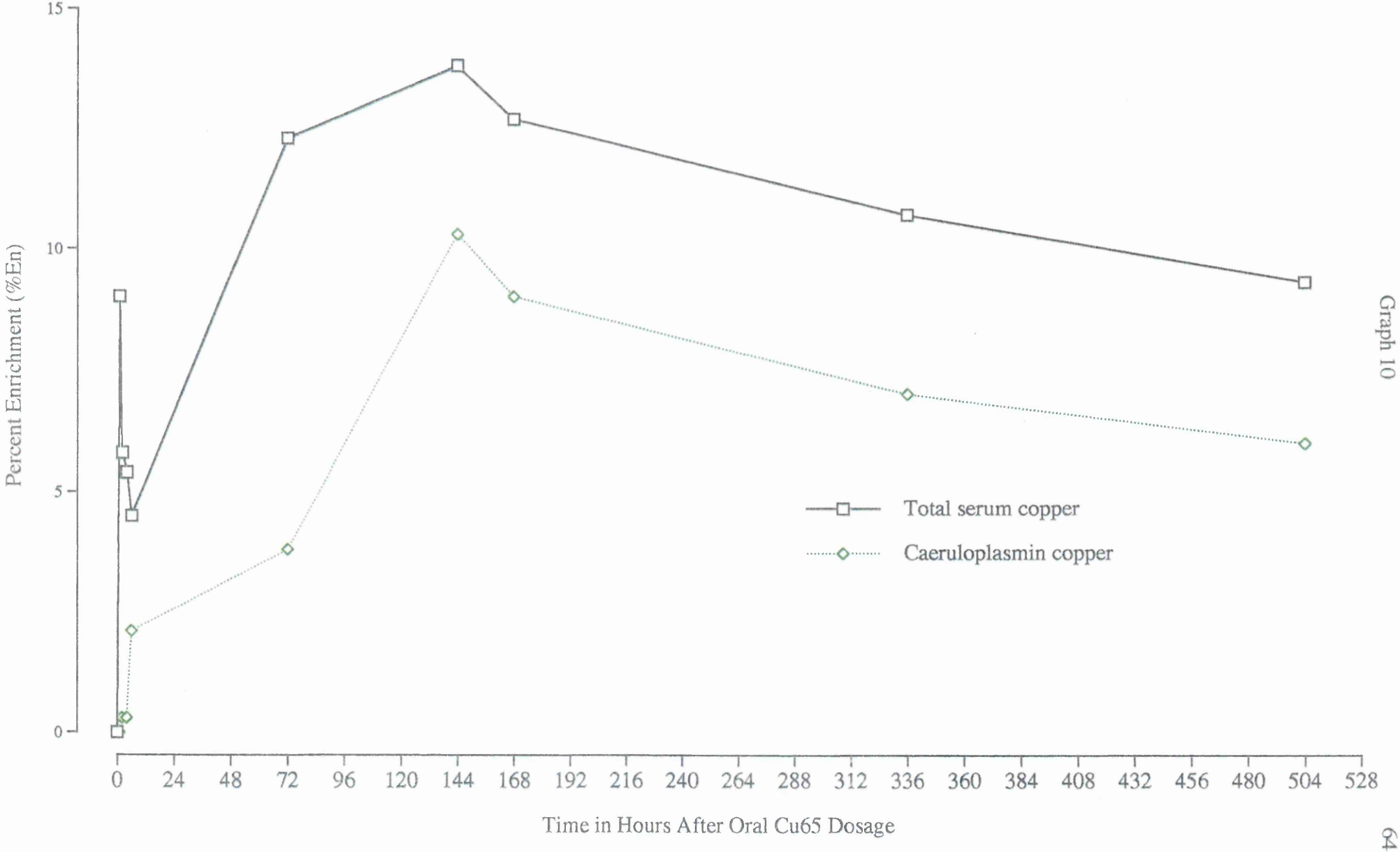
The mean %En ex-FPLC = 205. Mean %En post-FPLC = 138. This gives a 33% loss of enrichment following chromatographic separation. Two points are notable, first that %En is independent of Cu concentration and secondly that there is less than 2% variation in the degree of exchange. Despite higher enrichments on the FPLC isolated Cp and different degrees of labelling, the results conclusively demonstrate a consistent loss of approximately 30%En post-FPLC.

APPLICATION TO WD KINDREDS

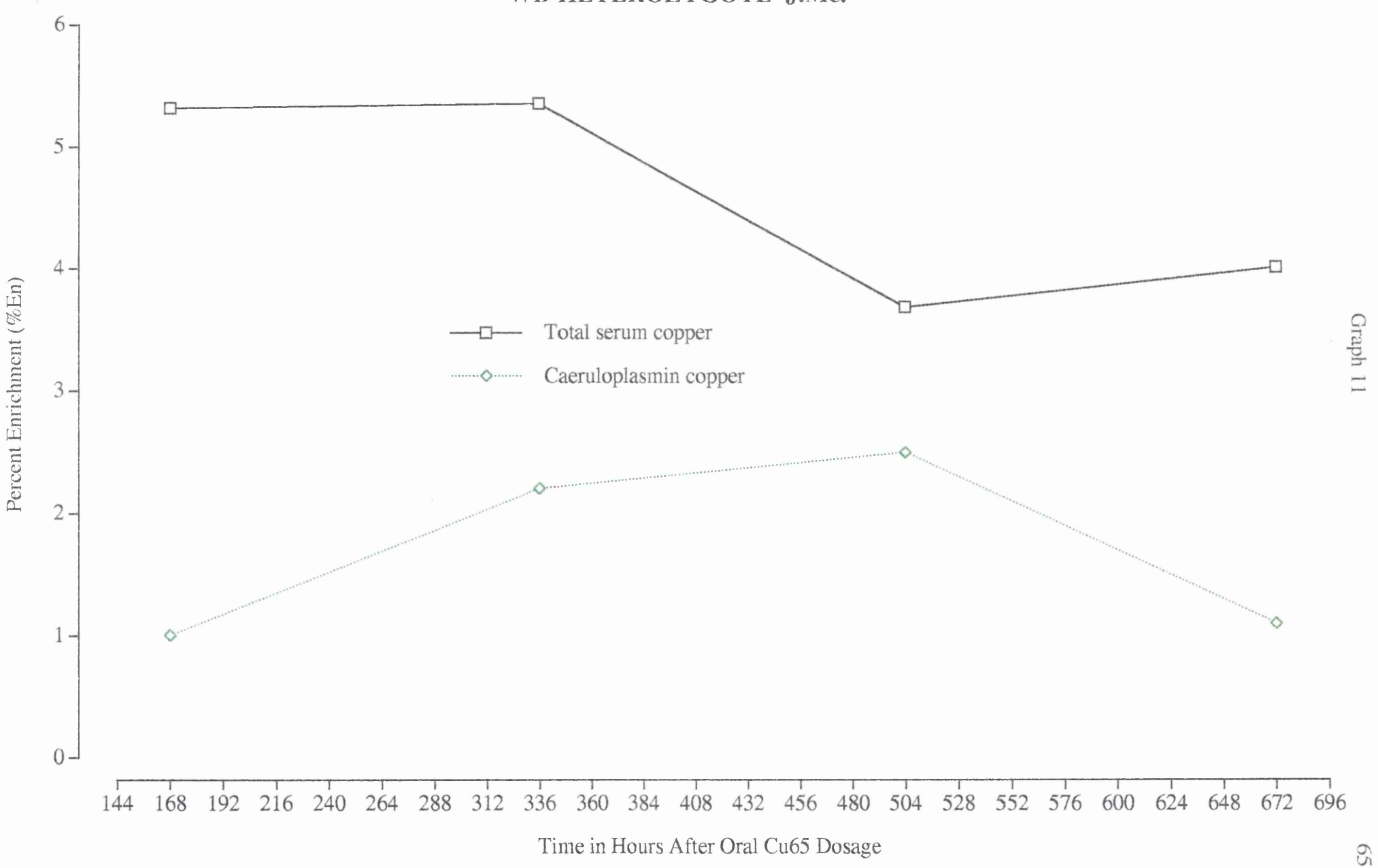
The application of FPLC isolation of Cp in Wilson's Disease kindred's was investigated. The subjects studied were heterozygotes or unaffected. WD cases have a low total serum Cu and Cp and do not incorporate ^{65}Cu effectively, preventing their study by this procedure. The first subject, S.A., an unaffected female with two affected brothers showed a normal enrichment (Graph 10)

with levels similar to S.M. in the metabolic study. She was followed over a four week period with blood samples withdrawn at 0, 1, 2, 4, 6, 72 hrs and 1, 2, 3 and 4 weeks. The other subjects are three obligate heterozygotes from the same family sampled at 1, 2, 3 and 4 weeks after oral dosage (Graphs 11,12,13). As we would expect, the enrichments are lower than the values obtained for normals in the metabolic study. An interesting observation is the later occurrence of a peak in Cp enrichments. In the four normals this is one week after ingestion of ^{65}Cu whereas the three heterozygotes have peak enrichments two or three weeks after the dose. This may be indicative of a slower or partially defective mechanism for handling copper in those who do not develop the full-blown disease.

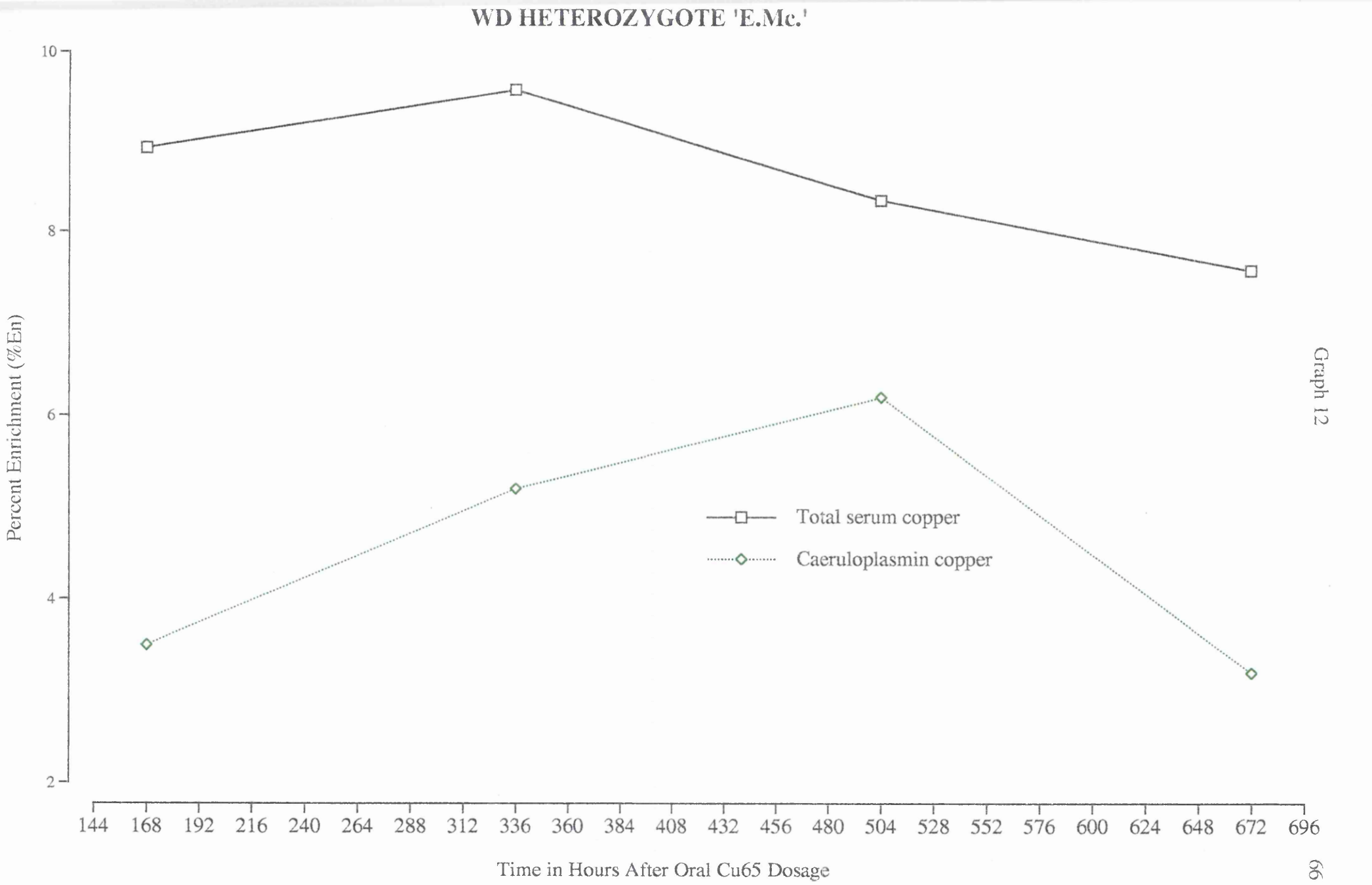
WD KINDRED NORMAL 'S.A'



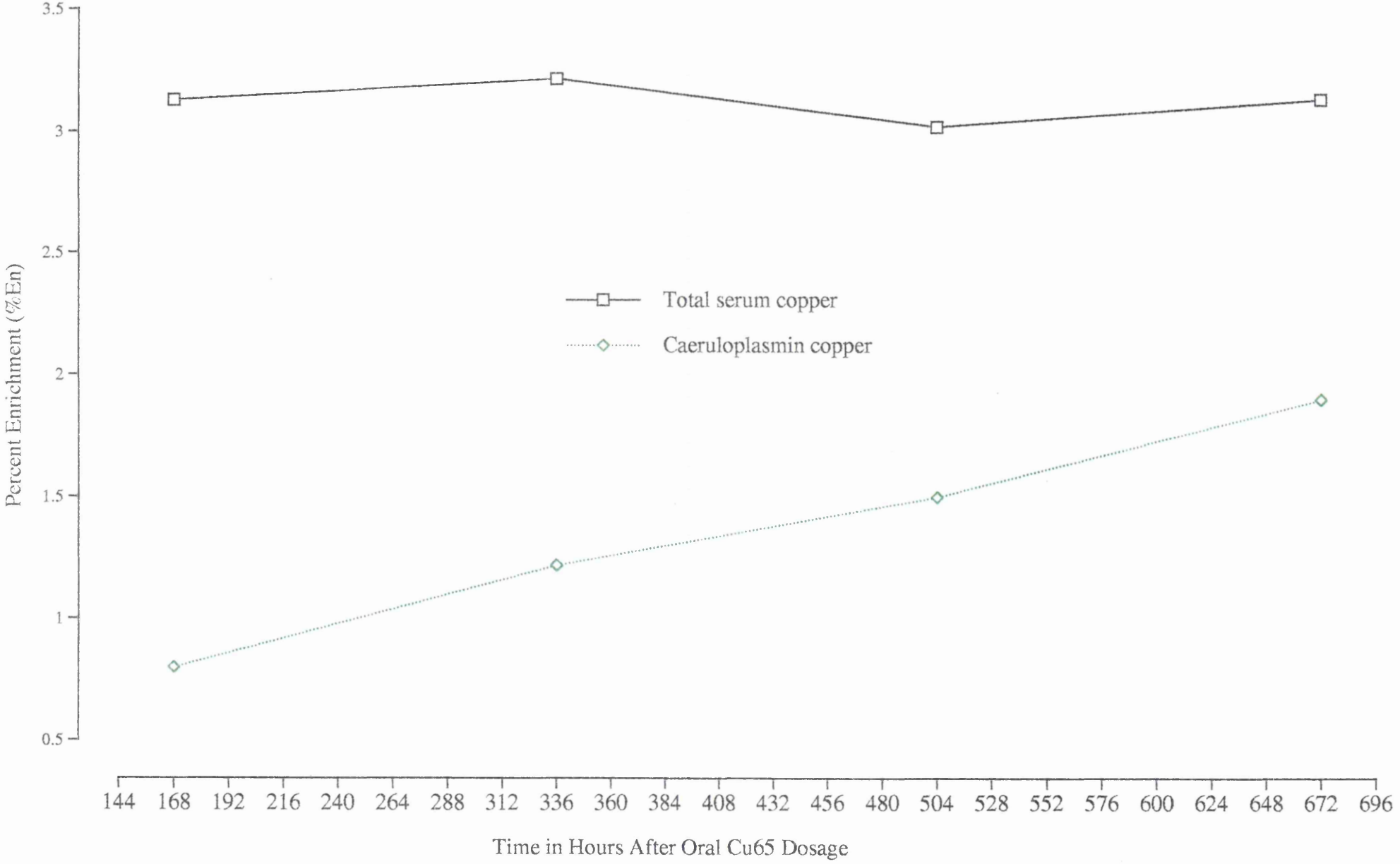
Graph 10


WD HETEROZYGOTE 'J.Mc.'
Percent Enrichment (%En)
0
1
2
3
4
5
6
Total serum copper
Caeruloplasmin copper
144 168 192 216 240 264 288 312 336 360 384 408 432 456 480 504 528 552 576 600 624 648 672 696
Time in Hours After Oral Cu65 Dosage

Graph 11


WD HETEROZYGOTE 'E.Mc.'
Percent Enrichment (%En)
10
8
6
4
2
Total serum copper
Caeruloplasmin copper
144 168 192 216 240 264 288 312 336 360 384 408 432 456 480 504 528 552 576 600 624 648 672 696
Time in Hours After Oral Cu65 Dosage
Graph 12

WD HETEROZYGOTE 'T.Mc.'



Graph 13

AFFINITY CHROMATOGRAPHY FOR CAERULOPLASMIN

TENTACLE SEPHAROSE

A chromatographic media with a particular affinity for caeruloplasmin was first described in Italy (Calabrese et al 1988). The group produced a derivative of Sepharose 4B by reaction with chloroethylamine. The resulting polyethylenimine is a branched polymer with an amine group distribution of 25% primary, 50% secondary and 25% tertiary. This has the property of selectively binding caeruloplasmin, and only a few other proteins.

Calabrese et al were interested in the electroparamagnetic (EPR) properties of the three distinct classes of copper-binding sites in caeruloplasmin. Type 1 copper occupies a mononuclear intensely blue site and the type 3, binuclear site is EPR-undetectable because of magnetic pairing. Type 2 is the most important for EPR studies. However, conventionally isolated Cp undergoes conformational changes leading to irreversible modifications in EPR spectroscopy. To visualise the copper in its natural state required the isolation of Cp by a rapid and single-step procedure. This allowed the demonstration of a dramatic change in the EPR copper signal due to physiological aging. The EPR signal for Cp-copper was stable for individuals up to 65 years old and then increased two-fold in older subjects. It is well known that modifications of protein structure occur during growth and development, but that further changes occur in ageing was previously unobserved.

I used the tentacle gel to follow the enrichment of albumin with ^{65}Cu, in addition to total serum and caeruloplasmin enrichments. This was previously prevented by the removal of Cu from albumin by the DEAE gel, during the FPLC seperations.

The 'tentacle gel' used is as prepared by the Italian group as follows;

Sepharose 4B (Pharmacia) in a 300ml gel bed suspended in 100ml of 5M NaOH, was treated with epichlorohydrine (25mls) at 70°C for two hours, and then, after washing with 10M NaOH, with 130mls of chloroethylamine at 70°C for two hours. A pH of 9-10 was maintained by addition of 10M NaOH. The derivatized Sepharose 4B was thoroughly washed with distilled water and equilibrated with 30mM phosphate buffer pH6.8 .

USE OF 'TENTACLE GEL'

1. All operations should be carried out in a cold room at 4°C.
2. Load 1 to 2mls of the gel slurry onto a column (0.5 cm x 20 cm) and let it sediment. This takes about 30mins. Note; The gel is not stable enough to withstand applied pressure from a pump and is run under gravity.
3. Wash the gel using two bed-volumes of NaOH (100mM). This will eliminate bacterial growth and protease activity. Then wash with deionized water to neutral pH.
4. Wash with two bed-volumes of MES (morpholinethanolsulphonic acid) 150mM, pH 6.7
5. Load the gel with 4ml of plasma or serum adjusted to pH 7.4
6. A blue band appears on top of the gel column.
7. Wash with MES buffer to eliminate traces of albumin. Monitor UV absorbance at 280nm.
8. Elute Cp using di-sodium phosphate buffer (pH 7.2),(300 - 500mM).
9. The column can be reused, after washing with NaOH. One ml bed volume can be used for 20ml serum or plasma.

TOTAL SERUM COPPER RECOVERIES

The copper recovered after separation of 2.5mls serum on tentacle gel was as follows:

Serum pool (50mls)	Serum Cu (µmol/l)	Cu conc. of isolated Cp (µmol/l)	% Recovery
A	24	23	96
B	20	19	95
C	20	18	90
D	22	20	91
E	15	13	86
F	21	20	95
G	23	20	86
		Average	**91**

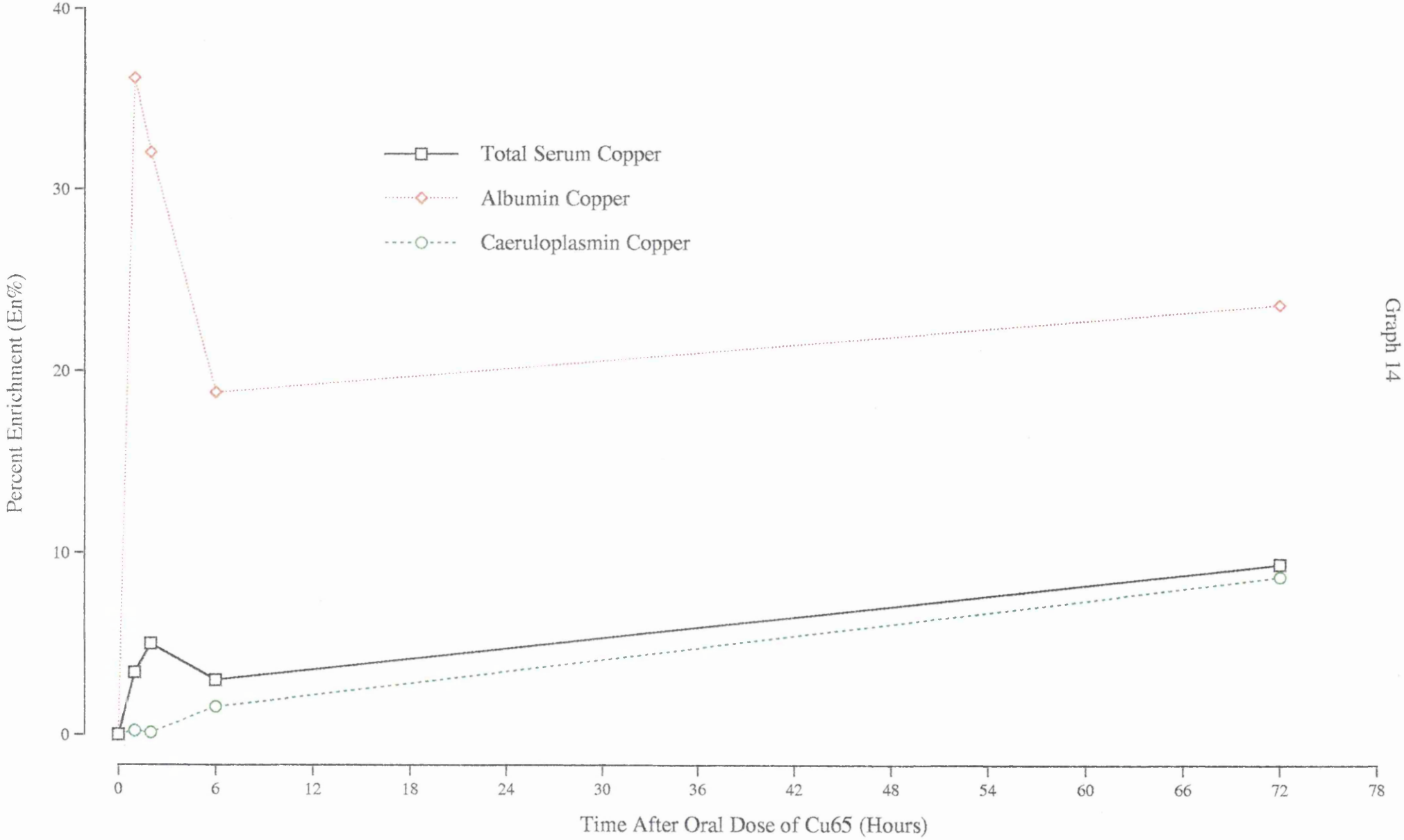
RECOVERY OF CAERULOPLASMIN

(measured by immunodiffusion)

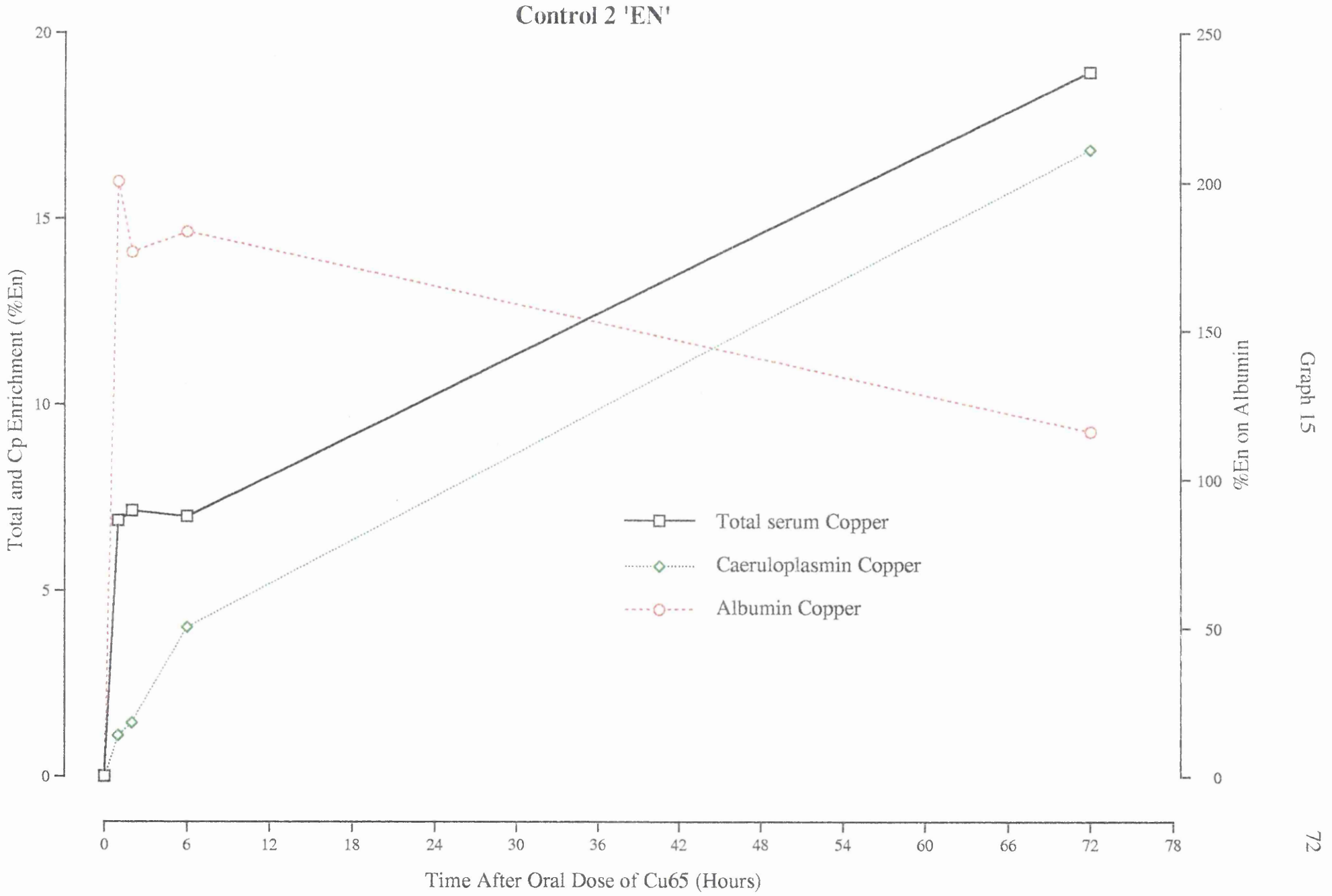
Serum pool (50mls)	Cp value (mg/l)	After separation	% Recovery
A	513	493	96
B	456	437	96

Four controls and one patient with malabsorption have been investigated using this separation procedure. Blood samples were withdrawn at 1, 2, 6 and 72 hours after ingestion of 3mg ^{65}Cu. The serum was separated and preserved with 20mM ε-ACA. Half the serum was used for total serum copper measurement. The remainder was separated on tentacle gel columns to isolate albumin and caeruloplasmin copper. The samples were prepared for ICP-MS measurement in the usual way. Results are shown in Graphs 14,15,16,17 and 18:-

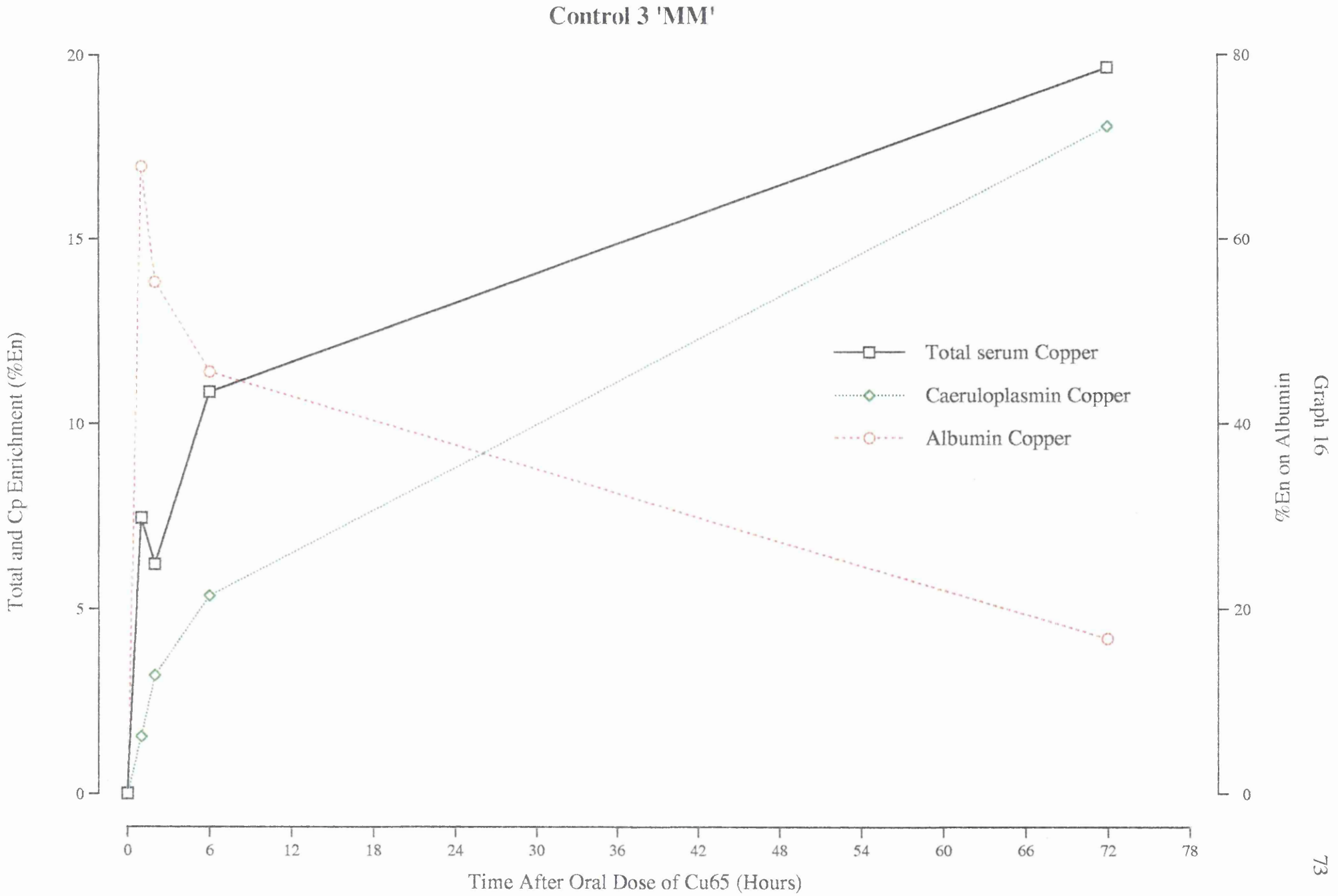
Control 1 'MS'



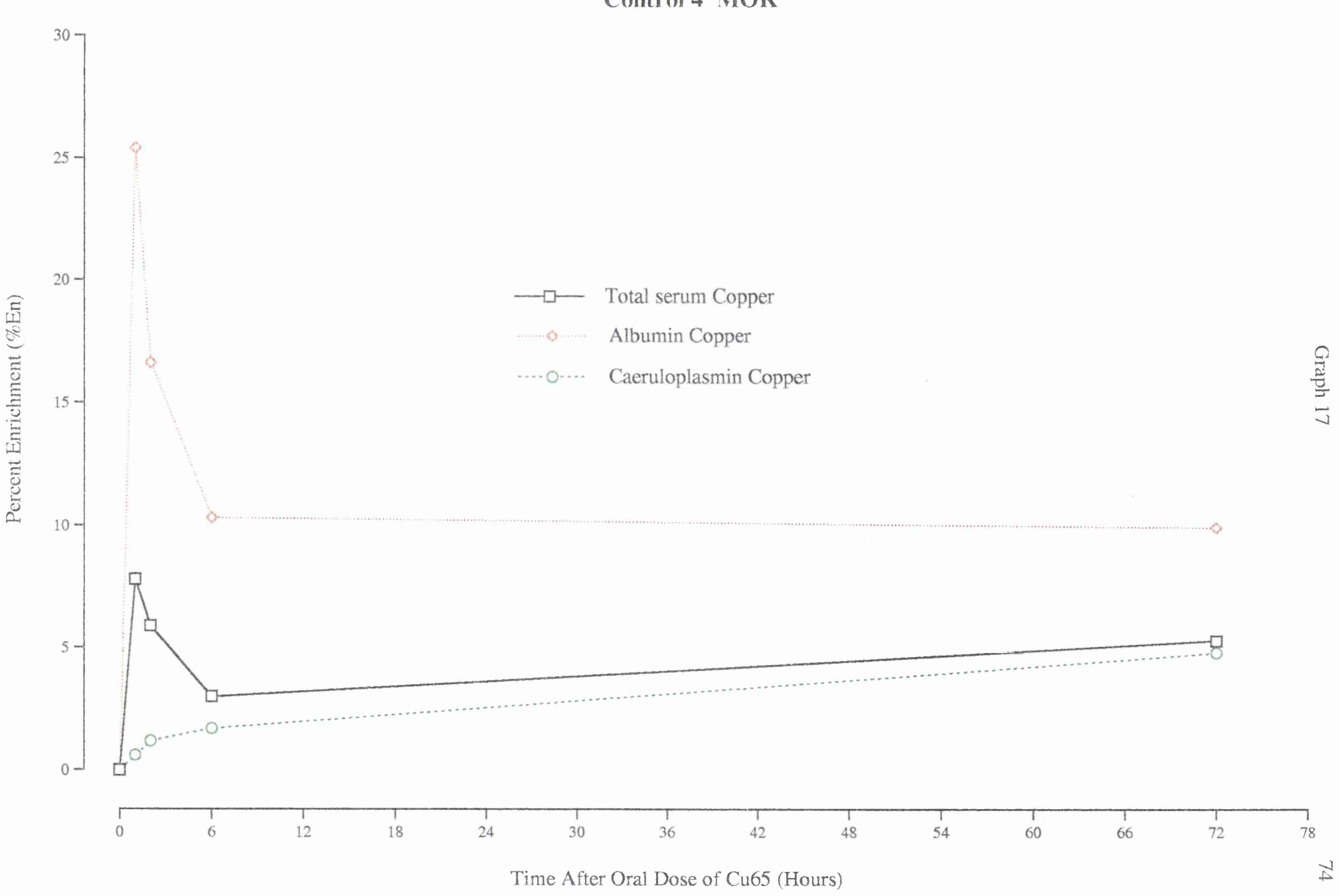
Graph 14


Control 2 'EN'
Total and Cp Enrichment (%En)
%En on Albumin
Time After Oral Dose of Cu65 (Hours)
Total serum Copper
Caeruloplasmin Copper
Albumin Copper
0
5
10
15
20
0
50
100
150
200
250
0
6
12
18
24
30
36
42
48
54
60
66
72
78

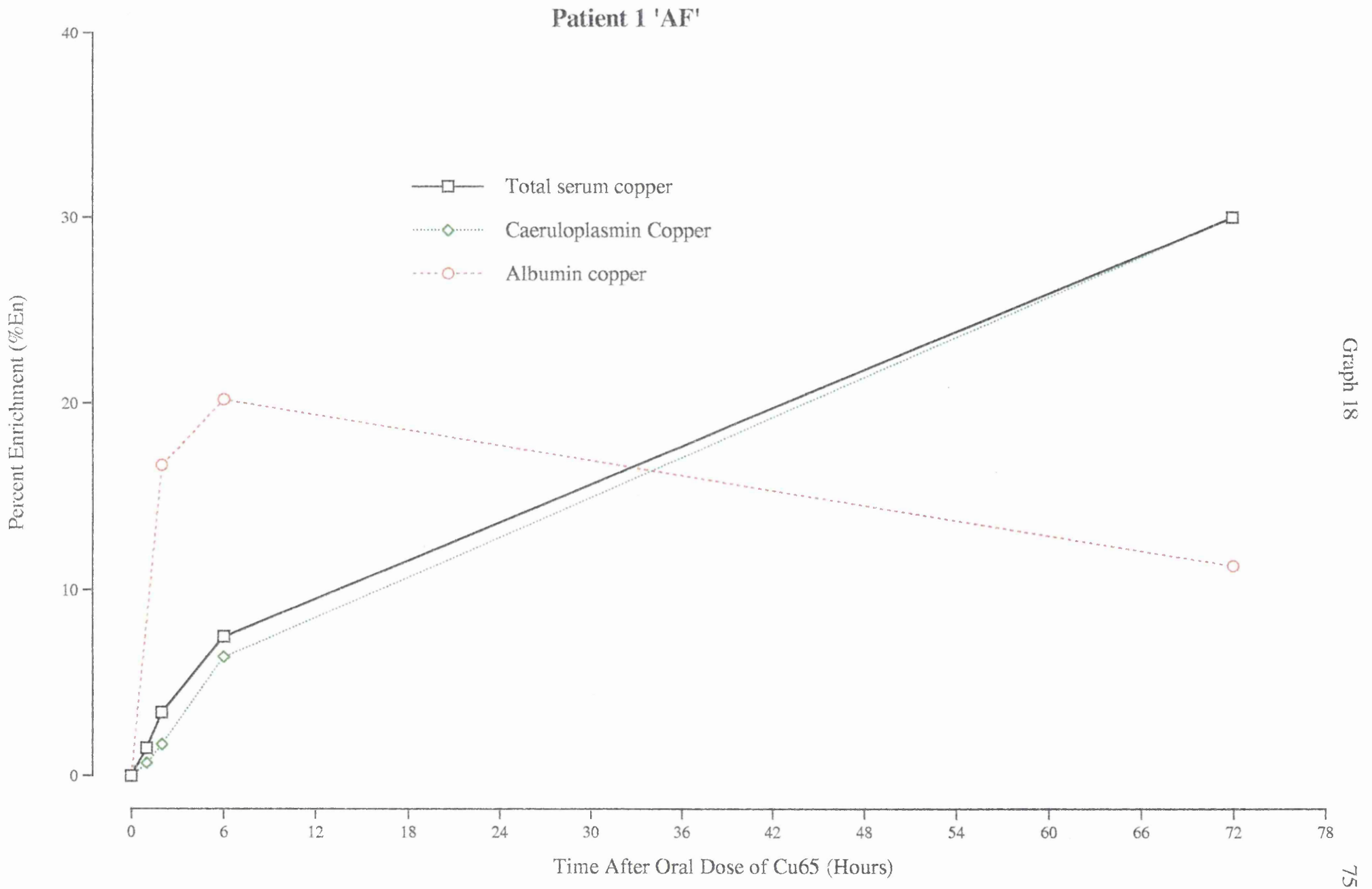
Graph 15


Control 3 'MM'
Total and Cp Enrichment (%En)
%En on Albumin
Time After Oral Dose of Cu65 (Hours)
Total serum Copper
Caeruloplasmin Copper
Albumin Copper
0
5
10
15
20
0
20
40
60
80
0
6
12
18
24
30
36
42
48
54
60
66
72
78

Graph 16


Control 4 'MOK'
Percent Enrichment (%En)
30
25
20
15
10
5
0
Total serum Copper
Albumin Copper
Caeruloplasmin Copper
0
6
12
18
24
30
36
42
48
54
60
66
72
78
Time After Oral Dose of Cu65 (Hours)

Graph 17


Patient 1 'AF'
Total serum copper
Caeruloplasmin Copper
Albumin copper
Percent Enrichment (%En)
40
30
20
10
0
0
6
12
18
24
30
36
42
48
54
60
66
72
78
Time After Oral Dose of Cu65 (Hours)

Graph 18

DISCUSSION

The four controls show the previously observed pattern of ^{65}Cu enrichment in total serum and caeruloplasmin copper. Enrichment of albumin-Cu is seen to coincide with the initial rise in total serum enrichment. The subsequent drop in Alb-Cu %En follows an increase in total serum and Cp %En. This fits in with our preconceived ideas of how ingested copper is handled. The large %En seen on Alb reflects the relative proportion of serum copper bound to this protein. As Alb-Cu accounts for < 5% and Cp-Cu up to 95%, so the ^{65}Cu dose received has a larger effect on the smaller amount of Alb-Cu in serum. This effect cannot be viewed in isolation as it is influenced by a number of other factors. The %En of Alb-Cu occurs when ^{65}Cu absorbed by the intestinal mucosa enters the liver via the hepatic portal vein, is not initially stored and appears in the peripheral blood. In health this is influenced by the liver's capacity to store the copper or it's immediate requirement for it, which in turn depends upon the tissue distribution, serum Cu and Cp as well as body weight and surface area of the individual.

The Crohns patient with intestinal malabsorption (Graph 18, Diag.13) has a different pattern of enrichment. There is no initial rise in total serum enrichment and low Alb-Cu %En. The initial peak at 1-2hrs is usually attributed to the normal overflow of Alb-Cu from the liver. It's absence suggests that only a small proportion of the given dose was absorbed. This was confirmed by measurement of faecal copper which showed a high proportion of ^{65}Cu appearing immediately in the excreta (Diag.13). There is incomplete recovery of pellets after seven consecutive collections which may be due to altered gut motility. The patient does not have any problem incorporating Cu into Cp. It appears that Cp accounts for all the serum Cu in this individual as Cp %En equals total serum %En after 72hrs. This may be due to low Alb concentrations or loss of the Alb-Cu binding site.

COPPER-65 METABOLIC STUDY-FAECAL SAMPLES

A.F

Code	Pellets	Total Dry Wt.	(65/63)m	(65/63)0	Sample Wt.	Vol.	Conc.	Faecal Cu conc.	Total Cu	Cu-65 tracer	Cu-65 tracer
		g			g	ml	µmol/l	µmol/g(dry)	µmol	µmol	mg
AF1	0	6.5	0.8859	0.4474	0.1	20	3.70	0.7400	4.8100	1.1184	0.0711
AF2	3	40.9	1.8093	0.4474	0.1	20	7.00	1.4000	57.2600	27.7587	1.7641
AF3	0	1	1.2372	0.4474	0.1	20	3.90	0.7800	0.7800	0.2754	0.0175
AF4	4	6.5	0.967	0.4474	0.1	20	5.10	1.0200	6.6300	1.7514	0.1113
AF5	2	9	0.6468	0.4474	0.1	20	1.70	0.3400	3.0600	0.3705	0.0235
AF6	4	25.7	0.5551	0.4474	0.1	20	2.1	0.4200	10.7940	0.7475	0.0475
AF7	5	51.7	0.4818	0.4474	0.1	20	0.7	0.1400	7.2380	0.1680	0.0107
									TOTAL EXCRETED	32.1899	2.0457
	18 out of 20 recovered								%RETENTION	**31.8**	

Key

m = measured ratio

0 = natural ratio

CONCLUSIONS

Having demonstrated the pattern of ^{65}Cu incorporation into whole serum, it remained to be seen whether the technique could be refined to look at individual proteins. The investigation began with gel filtration chromatography. This proved to be slow and inefficient in separating the main copper protein fractions. Other methods were partially effective but were correspondingly flawed. PEG precipitation is limited to a preliminary purification step while affinity chromatography of albumin takes time and was often inconsistent. It was in ion exchange chromatography that a practical solution was found. The DEAE anion exchange gel provided the protein capacity and physical stability to allow the high flow rates used with FPLC. This combination was used to separate caeruloplasmin from serum samples taken at timed intervals post oral ^{65}Cu dosage in four controls. Although there was some loss of ^{65}Cu enrichment during the separation procedure this was accounted for and an important conclusion could then be made. That is, that the secondary rise in total serum ^{65}Cu enrichment closely mirrors that of chromatographically isolated caeruloplasmin. This procedure was subsequently used to investigate a single unaffected sibling from a WD kindred and three established WD heterozygotes.

A further refinement was made possible by use of 'tentacle' sepharose. Four control subjects were studied and ICP-MS measurements of ^{65}Cu on albumin confirmed that the initial rise of total serum enrichments at 1-2hrs was due to copper bound to this protein. The identification of two important copper-binding proteins of human serum resolves the components of total serum enrichments and may helps formulate a model of copper metabolism. Computer simulation of copper metabolism (Blincoe 1993, Watson 1995), is greatly enhanced by such information allowing estimates of whole body copper and tissue distribution. This should lead to better

discrimination between the numerous diseases associated with marginal copper deficiency and other disease (Danks 1991,Wierzbicki 1993, Logan 1994).

The applicability of the ^{65}Cu isotope to a wide range of research interests offers exciting possibilities. For cardiovascular health, copper is required as an important antioxidant (Suciu 1992, Allen 1994). When preterm babies and infants are receiving intravenous feeding with potentially Cu deficient regiments, ^{65}Cu may be used to determine the distribution and excretion of copper and thereby estimate true copper balance. In adults receiving intravenous feeding, or with severe malabsorption, the tracer may be used to determine copper status. Elderly people have changes in caeruloplasmin that isotope studies could clarify (Musci 1993, Semsei 1993). The general population might also benefit from studies on the bioavailability of copper using ^{65}Cu spiked diets (Milne 1993). The suggestion has been made that modern diets may be Cu deficient. The World Health Organisation (WHO) recommends 2mg/day Cu and this may not be reached for a number of reasons. Traditionally, animal offal (a good source of copper) was consumed in much greater amounts by the population as a whole. Copper in liver, tripe and sweetmeats is associated with metalloenzymes, proteins and amino acids in small stable complexes that are more readily absorbed than the free Cu^{++} ion (MAFF 1981). The modern trend is towards increased consumption of highly processed foodstuffs in which the structure and composition of ingredients is altered. It would be possible, by growing plants and rearing animals on ^{65}Cu feeds to compare the availability of copper from fresh and processed food.

The highly sensitive ICP-MS procedure is amenable to the study of $^{65/63}Cu$ ratios in proteins, plant and animal cells and tissues as well as organic material.

METHODS

METHOD 1

DESALTING AND BUFFER EXCHANGE

Since proteins differ greatly in size from salts and peptides, gel filtration is particularly efficient in the extraction of sodium chloride from, and exchange or removal of solutions surrounding proteins. It is necessary to remove salt to prevent precipitation of proteins and to adjust the sample to the correct ionic conditions for ion exchange or ICP-MS work. The solution surrounding the proteins may require adjustment for pH, storage or replacement with pure water.

For this the low MW cut-off of the gel media Sephadex (Pharmacia) is utilised. Sephadex is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. In its smallest dry bead size (G-25 Superfine) it has a diameter of 10-40μm supplied pre-packed (Fast Desalting Column HR 10/10). With a slightly larger diameter of 50-150μm (G-25 Medium) supplied pre-packed in disposable columns (PD-10). Both grades of Sephadex have a low MW cut-off of 1kDa and any particles smaller than this are trapped in the gel matrix.

The sample is applied to the columns according to the following procedures to remove more than 95% of salt originally present, elute the protein in pure water, terminate reactions or prepare it for subsequent chromatography.

PD-10 Columns

Prepacked 'PD-10' columns contain a preservative but should be checked before use for discoloration or drying. To achieve greater yields, samples are passed through the column twice.

INSTRUCTIONS

1. Pour off liquid on top of column and equilibrate with 30mls of required solution.
2. Load sample in a total volume not exceeding 2.5mls. Discard eluent.
3. Elute high MW components with 3.5mls solution - keep.
4. Re-equilibrate column with 30mls solution as before, taking the 2.5ml sample to be loaded from the 3.5mls already eluted.

Fast Desalting Column HR 10/10 on FPLC

1. Sample applied via Superloop in a maximum volume of 1ml.
2. Sample run at 1ml/min.
3. Eluted in 2-3ml volume in 1 min.
4. Re-equilibrate with 20mls required solution.

METHOD 2

SPECTROPHOTOMETRY

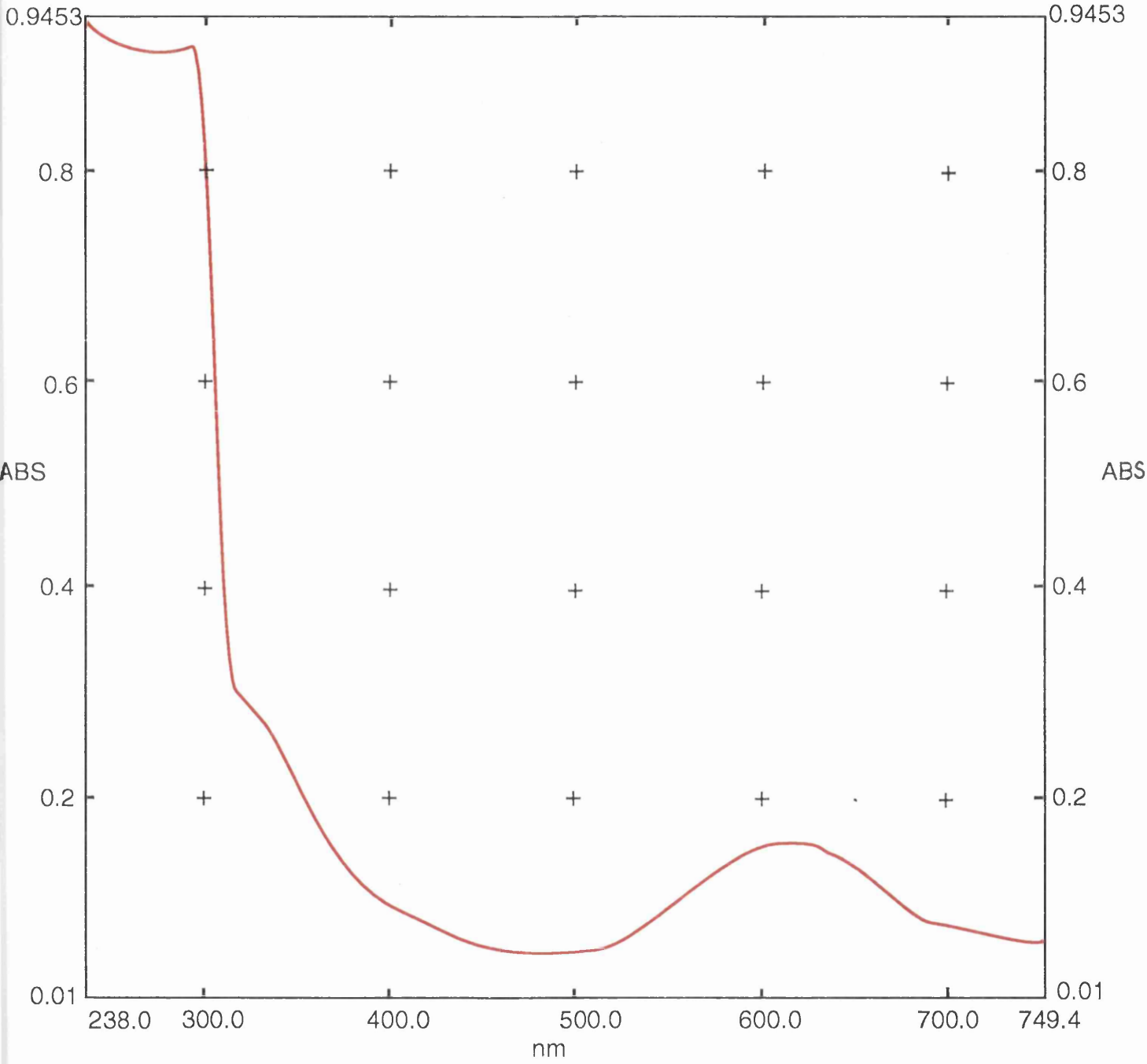
Measuring the light absorbed by the column effluent at 280nm is a continuous and automatic method for monitoring a chromatographic separation. It depends upon chromophores in the protein such as the peptide bond, amino acid side chains and prosthetic groups. Before collection, the effluent is passed through an optical flow cell housed in the detector.

Light passing across the flow cell is registered by a photodetector and compared to a reference cell. The detector gives a response when an absorbing compound passes the cell. The response signal is plotted on a pen recorder to document retention time and resolution.

Our protein of interest caeruloplasmin, possesses the additional quality of being coloured. This allows us to measure the relative purity of eluted fractions. Fractions as small as 1ml are measured in a spectrophotometer (Philips, Beckman) at 610nm against a pure water blank. By measuring the absorbencies at 280 and 610nm of completely pure caeruloplasmin, a corresponding ration is produced. This is derived from the absorbance at 610nm divided by the absorbance at 280nm. A 90% pure caeruloplasmin preparation gives a ration of 0.045 or better (Arnauld 1988). A scan from 280nm to 610nm of such a preparation is presented in Diag.14;

Diag. 14

OPTICAL SPECTRUM OF CAERULOPLASMIN



METHOD 3

SOLVENT EXTRACTION

Samples for ICP-MS work require removal of sodium (Na) to less than 10ppm. Desalting by gel filtration may not always achieve this due perhaps to air bubbles or channels in the gel bed.

However, by forming a complex with a reagent and removing it into a separate solvent phase complete removal of sodium is ensured. The reagent used is ammonium pyrrolidinedithiocarbamate (APDC). It is obtained as a white crystalline substance of MW164.3.

APDC forms complexes with more than 30 elements and most of these complexes can be extracted into organic solvents. The complex with copper forms at pH 0-14 and is extracted with methyl isobutyl ketone (MIBK). The wide range of metals complexed by APDC together with the stability of the reagent in acid solution, make it very useful general extracting reagent for heavy metals. It may be used in the following ways:

(a) The concentration and/or separation of heavy metals prior to their determination by atomic absorption spectrophotometry.

(b) As a qualitative reagent for the identification of metals.

(c) As a reagent for the determination of metals by spectrophotometry in the visible or ultra-violet regions.

(d) As a means of removing heavy metals before the EDTA titration or spectrophotometric determination of elements such as magnesium, calcium, strontium, barium, aluminium or the lanthanons.

The protocol for ICP-MS samples is as follows:

(1) APDC solvent extracted with MIBK three times to remove any trace metals in the APDC.

(2) Two drops Thymol Blue 2% (a pH indicator) to each sample and 7mls of deionised water.

(3) Concentrated ammonia until greenish-yellow.

(4) Add 2mls APDC shake vigorously for 2 minutes.

(5) Add 2mls MIBK shake vigorously for 2 minutes.

(6) Remove solvent layer with pastette and heat at 150^{0}C in an acid washed 50ml pyrex beaker.

(7) Cool to room temperature.

(8) Dissolve the dry extracts in 0.25ml of concentrated nitric acid followed by 0.75mls of deionised water.

(9) Once complete dissolution is ensured, add 8mls of deionised water, mix by inversion several times and store at 4^{0}C until analysis.

METHOD 4

ELECTROPHORESIS

The most reliable way to determine the purity or contaminants of a given sample is by electrophoresis on agarose gel. A single band is usually indicative of 100% purity. For convenience, an electrophoresis kit 'Hydragel High Resolution' (Sebia) was used. The composition of the gel gives better resolution than a standard agarose gel. Instead of five or six fractions, this gel gives about ten or more fractions. An example is given in Diag. 15;

REAGENTS

Running Buffer:	Tris	7.2 g/l
	Barbital	1.8 g/l
	Sodium Barbital	10.3 g/l
	Sodium Azide	0.1 g/l
Stain:	2 g/l of acid violet in 10% acetic acid	
Fixative Solution:	Ethanol	60%
	Acetic acid	10%
	Distilled water	30%
Destain Solution:	Acetic acid	10%

PROCEDURE

(1) Dilute the serum samples 1:10 with saline. The biological fluids with low protein concentration must be concentrated in order to obtain about 6 g/L, of proteins.

(2) Remove the gel from its packaging, just before sample application.

(3) Blot quickly the excess of liquid on the gel surface, with a narrow strip of filter paper.

(4) Place the sample template, level with the arrows. Make sure there are no air bubbles between the sample template and the gel.

(5) Apply 5µl of sample on each slit. Let it diffuse for 5 minutes after last sample has been

applied. Blot the excess sample with a template blotter.

(6) Remove the sample template.

(7) Place the gel in the tank respecting the anodic and cathodic sides.

(8) Plug the tank to the power supply and run the separation.

Running conditions are as follows:

Tank	**Sebia K20***
Volume of buffer per compartment	150ml
Total	300ml
Voltage (constant)	100 V
Starting current per gel	23 mA (+/-2)
Migration time	35 min

* Tank K20: the gel is positioned upside down and dips 1cm into the buffer on each side.

(9) After migration, unplug the tank and remove the gel.

(10) Rinse quickly with distilled water.

(11) Immerse the gel vertically in the fixative solution for 15 min, in the washing rack.

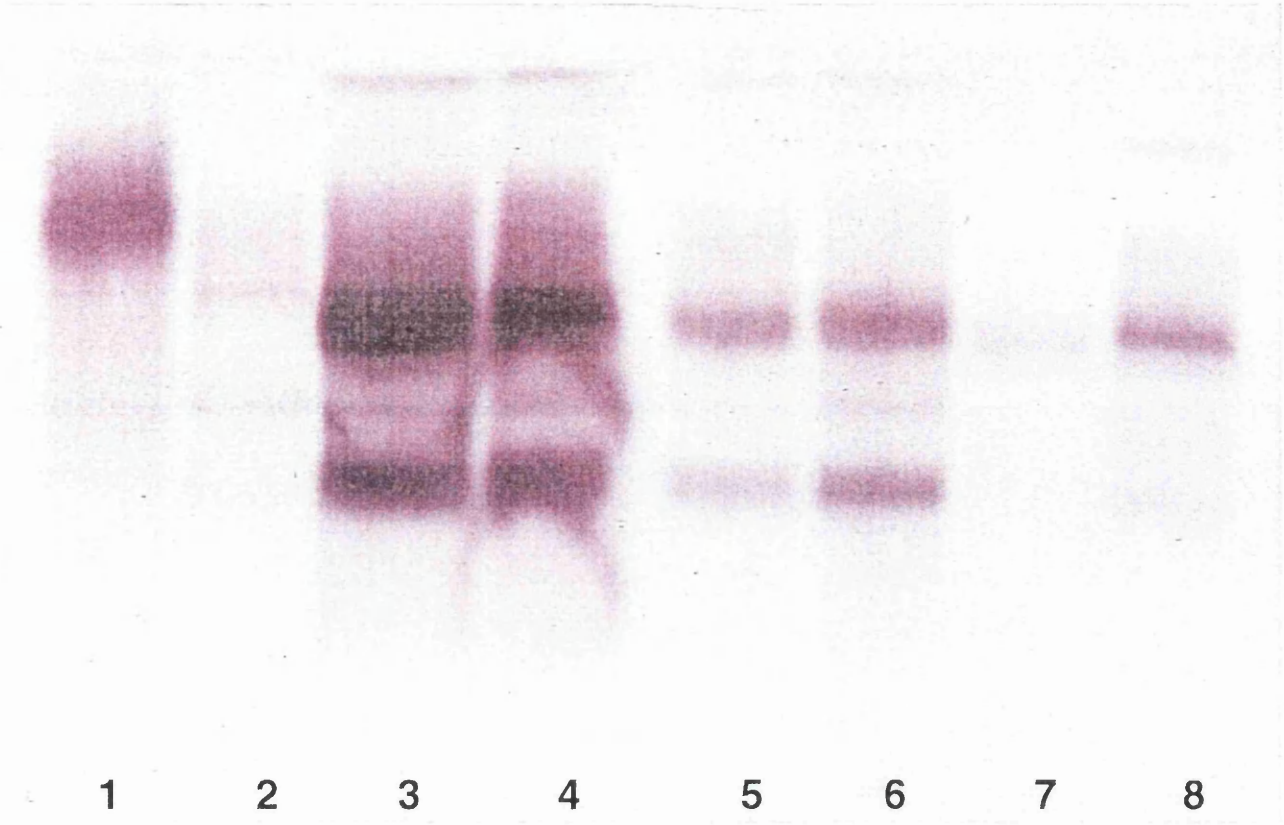
(12) Remove the gel from fixative and dry it with hot air at more than or equal to 60^0C.

(13) Immerse the dry (and cooled) film in the staining solution for 15 min.

(14) Destain in two successive baths of destaining solution, until the background is completely clear.

(15) Soak up excess liquid on the gel surface with a thin filter paper and dry the film with hot air approximately 60^0C.

ELECTROPHORESIS EXAMPLE



1 2 3 4 5 6 7 8

Key

Lane 1 : Unbound Fraction post DEAE ion exchange

Lane 2 : bound Fraction post Mono Q ion exchange

Lane 3 : bound Fraction eluted from DEAE gel

Lane 4 : bound Fraction eluted from DEAE gel

Lane 5 : bound Fraction eluted from Tentacle Sepharose

Lane 6 : bound Fraction eluted from Tentacle Sepharose

Lane 7: Single Cp band after FPLC Tentacle gel

Lane 8 : Commercial Cp (Sigma)

METHOD 5

COPPER BY GRAPHITE FURNACE

It was necessary, where samples contained less than 5μmol/l copper to use graphite furnace atomic absorption because of it's greater sensitivity. This method is adapted from a routine method for paediatric serum sar. ples and was used without any quality control (QC). Copper concentrations were only measured to determine the dilution required for the ICP-MS. The method requires a 20μl sample only. Care must be taken to avoid contamination. Acid washed pipette tips are used in the preparation of all reagents and standards.

PROCEDURE

Into acid-washed autoanalyser cups, pipette 200μl of 1% Triton buffer. Add 20μl of standard or sample. Mix on a vibro mixer and load cups onto graphite furnace autosampler.

INSTRUMENT CONDITIONS

For Perkin Elmer 2280 with HGA400 and AS40 autosampler:

Wavelength	325nm	Injection volume	20μl
Slit width	0.7nm	Chart recorder	5mV
Background correction	ON	Chart speed	20mm/min
Lamp current	10mA	Scale Expansion	X2

CALCULATION OF RESULTS

Subtract the peak height of the O std from the peak heights of the standards. Subtract the reagent blank value from the peak height for the samples. Calculated using the Trace Element Calculation Programme (CALGRAPH), to the nearest 0.1μmol/l.

METHOD 6

COPPER BY FLAME AA

Samples containing copper in the range 5-30μmol/l were measured by flame atomic absorption. Copper concentrations were used to determine the approximate dilution required for ICP-MS ratio measurement. An appropriate QC was not available. This method requires a 300μl sample. No special precautions to avoid contamination are required, although the sample uptake tubing should be wiped with tissue between samples.

PROCEDURE

Samples are aspirated directly into the flame by hand and held until the read time has elapsed.

INSTRUMENT CONDITIONS

For GBC 904AA.

Wavelength	324.7nm	Acetylene Flow	2 units
Slit width	0.5nm	Air Flow	10 units
Background connection	OFF	Read Time	3s
Lamp current	4.0mA	Time Constant	0.5s

CALCULATION OF RESULTS

The instrument requires calibration before each run. Four standards are read and a calibration curve with the maximum error and R^2 fit automatically produced. When the maximum error is 0.5μmol/l and the R^2 fit is 0.999 or better, the samples are run without further recalibration. Results are automatically calculated and printed during the run.

METHOD 7

PROTEIN DETERMINATION

Caeruloplasmin concentrations were determined by single radial immunodiffusion (Behring). The agar plates contain monospecific antisera obtained by immunising rabbits. They are supplied in sealed aluminium containers containing 12 tests to be stored at 4^0C.

The controls and samples are introduced undiluted into wells in the plate. The volume required per well is 5µl. A 'Hamilton' micolitre syringe was used for this purpose.

After a diffusion period of 2 days at 4^0C the diameter of the precipitates was measured with the NOR-Partigen measuring template. This is accurate to the nearest 0.1mm. Having previously obtained false results due to outdated plates some form of quality control (QC) was required. The accuracy is checked by means of control plasma for NOR-Partigen. The batch dependant precipitate ring diameter given in the table of assigned values accompanying the pack must lie within the confidence range (D = +/- 0.3mm)

The plate will measure from 70-1060mg/L caeruloplasmin.

METHOD 8

PROTEIN CONCENTRATION

Column fractions were pooled and concentrated with a static concentrator (Nanosep). This is a self-standing, easy-to-use, fast and reliable system of sample enrichment prior to electrophoresis or further chromatography.

SPECIFICATIONS

Cut off molecular weight (Daltons)	10,000
Cells per unit	4
Cell capacity per unit	10 ml
Maximum concentration factor	200 x
Deadstop, minimum recovery	50 µl
Size (w x l x h)	37 x 100 120 mm

INSTRUCTIONS FOR USE

1. Introduce the sample through the top of each cell with a pasteur pipette or syringe.
2. If containment is required, remove backing from flap and seal the cell.
3. Concentration will proceed completely unattended. When desired concentration is reached, open cell and withdraw concentrate with the Intersep pipette.
4. Slow concentration in fridge at 4^0C.

BENEFITS

- Capable of concentrating protein solutions up to 200x.
- Large capacity 10ml cells, saving time, no refilling.
- Unique disposable pipette for easy withdrawal of concentrate.
- Highly visible concentration levels and volume.
- Non-protein binding membrane developed by Hoechst AG for Intersep allows rapid concentration.

- Highly hydrophilic membrane surface allows complete product recovery.
- Cell can be sealed for odour containment, prevents evaporation.
- 50µl dead stop avoids sample desiccation.
- Desalts sample, >40% NaCl removed.

METHOD 9

ION EXCHANGE WITH CHELEX

Chelex-100 (Bio-Rad) was used to remove loosely bound copper from caeruloplasmin after labelling with Cu65 (Van Berkel 1988). The chelex (0.4g) was placed into 5ml plastic tubes and 2mls 2M nitric acid added. This was mixed on a roller mixer for 5mins to strip the resin of any Cu present. The tube was then spun in a benchtop centrifuge (3,000 rpm for 5 mins), and the supernatant removed by pipette. The resin was converted to its ammonical form by addition of 2mls 2M ammonia and mixing as before.

Conditioning was performed with 2mls 100mM sodium acetate buffer pH5.5. This was done three times before the pH of the supernatant attained 5.5.

Incubated Cp in 2.5mls sodium acetate buffer was mixed with the prepared resin for 10 mins on the roller mixer. After centrifugation as described the supernatant was removed by pipette and prepared for ICP-MS analysis or further chromatography by FPLC.

METHOD 10

SODIUM MEASUREMENT

Sodium concentration is measured prior to ICP-MS analysis to avoid false results caused by sodium-argon dimer formation. The sample must contain less than 10ppm sodium to prevent interference.

The instrument used was the ATOMSCAN25 (Thermo Jarrell Ash), a sequential plasma emission spectrometer. The emission source is an inductively coupled argon plasma which provides the ideal excitation and is inherently stable over long time periods.

PROCEDURE

Standardisation is carried out by a two point uncalibrated method. A blank and a sodium standard at 11.5ppm is all that is required as the instrument has an internal standard. Samples are aspirated via a peristaltic pump into a nebulizer before passing into the plasma. The sample tube is handheld until the read time has elapsed.

INSTRUMENT CONDITIONS

Wavelength	589.592	Flush pump rate	200rpm
Radio frequency	27.12 MHz	Analysis pump rate	100rpm
Background correction	YES	Flush time	30s
Torch gas flow	HIGH	Analysis time	36s

RESULTS

Results are automatically printed during the run in ppm. These are given as the average of two repeats with a percentage standard deviation. Standard deviations below 0.5% are acceptable.

METHOD 11

COPPER ISOTOPE RATIOS BY ICP-MS

The Quadropole ICP-MS was operated under the following conditions :

Plasma:

R.f. power	Forward	1.37 kW
	Reflected	<10 W
Gas controls	Auxiliary	0.61 min^{-1}
	Coolant	131min^{-1}
	Nebuliser	0.71min^{-1}

Torch ... ... Fassel type, cooling by tangential flow

Nebuliser ... ... Meinhart concentric type, solutions pumped at 0.7 ml min^{-1}

Spray Chamber ... ... Scott-type double bypass, water cooled

Ion sampling:

Sampling cone ... ... Nickel sampler (Nicone) with 1.0-mm orifice

Skimmer cone ... ... Nickel (001 Type) with 0.75-mm orifice

Sampling distance ... ... 10mm from load coil

Vacuum:

Expansion stage ... ... 2.4 mbar
Intermediate ... ... $<10^{-4}$ mbar
Analyser ... ... 3-4 x 10^{-6} mbar

Data acquisition:

Scan mode...	Sweeps	1600
	Dwell time	80 µs
	Channels	512
	Run time	65 s
	No. of runs	5
	Total time	5 mins 25 s

METHOD 12

OXIDASE ACTIVITY OF CAERULOPLASMIN

The most common technique for the determination of the oxidase activity is based on the measurement of the oxidation of the substrate p-phenylenediamine by Cp (Sunderman 1970, Saenko 1990).

PRINCIPLE

Spectrophotometric measurement of the rate of formation of the purple product following the oxidation of p-phenylenediamine (PPD) by Cp.

REAGENTS

Acetate buffer, 0.1 M, pH 5.45

Sodium azide solution 1.5 M

PPD (Sigma) solution, 27.6 mM in acetate buffer, pH 5.45.

PROCEDURE

Two millilitres of acetate buffer is mixed with 0.1 ml serum in a test tube and warmed at 37^{0}C. One millilitre of the PPD solution, also warmed at 37^{0}C is added. The tubes are mixed, and incubated for exactly 30 mins at 37^{0}C, protected from light. Fifty microlitres of sodium azide solution is then added, the reagents are mixed, and the sample is read in a spectrophotometer at 530nm. The colour is stable for two hours.

One of the sources of error when using PPD as a substrate is due to its enzymatic oxidation. To prevent this, a tube containing the same reagents, but to which 50µl of sodium azide (a irreversible inhibitor of the enzymatic oxidase activity) has been added at the beginning of the reaction is used as a control. The difference between this control and the reaction tube will determine the enzymatic oxidation of PPD. In addition, this PPD-oxidase reaction is subjected to a lag phase reportedly due to the oxidation by Cp of serum ascorbic acid. Therefore, the timing of the reaction is deliberately delayed and the control tube receives sodium azide 5min after PPD is mixed with the serum sample.

CALCULATION

Cp (mg%ml) = c(Absorbance of the sample - Absorbance of the control). Where c is a mean calibration factor determined by comparison with PPD-oxidase determination conducted with 'pure' Cp preparations and calculated at 75.2. Normal values are at 31.5 (+/- 4.9 SD) mg/100ml. With this method, the coefficient of variation is 1.25% and the day-to-day variability is 2.8%.

However, this procedure was not considered reproducible enough for quantification of Cp.

METHOD 13

CLEAN IN PLACE PROCEDURE (CIP)

Remove ionically bound proteins by washing the column with 0.5 bed volumes of a 2 M NaCl solution, contact time 10-15 mins, reversed flow direction.

Remove precipitated proteins, hydrophobically bound proteins and lipoproteins by washing the column with 1 M NaOH solution at a linear flow rate of approximately 40 cm/h, contact time 1-2 hours, reversed flow direction.

Wash with at least 3 bed volumes of starting buffer.

Remove strongly hydrophobically bound proteins, lipoproteins and lipids by washing the column with 4 bed volumes of up to 70% ethanol or 30% isopropanol, reversed flow direction. Apply increasing gradients to avoid air bubble formation when using high concentrations or organic solvents. Trace metals are removed with one bed volume of 200mM EDTA.

Wash with at least 3 bed volumes of starting buffer.

METHOD 14

METABOLIC STUDY SAMPLING PROCEDURE

1. Collect a 24 hour urine sample and a faecal sample. They do not have to be necessarily collected on the same day.

   For urine collection - Use bottles and funnel provided. After use, rinse funnel with tap water and dry with paper towel.

   For faeces collection - Collect urine first. Then collect stools (faeces) using the plastic container provided. It is important not to contaminate the faeces sample with urine.

2. Ensure diet includes plenty of fibres. Fibres are contained in vegetables, fruits, cereals, wholemeal bread and so on.

3. The experiment starts at 9.30 in the treatment room.

   Overnight fasting is essential.

   A 20 ml blood sample will be taken with a Venflon cannula, which will be left in the arm for the rest of the day to collect blood samples after the isotope ingestion.

   The following will be given:

   - two capsules of plastic pellets that will act as a faecal marker,
   - 3mg of copper (^{65}Cu isotope) in water.

   Eating will be allowed 3 hrs after the ingestion of the isotopes.

   Weight and height is recorded.

4. BLOOD SAMPLES

Besides the first blood sample taken before the experiment, 10-20 ml blood samples will be collected: 0.5, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5, 6.5 hrs after isotope ingestion.

A 20ml blood sample will be collected around 10.00am on Tuesday, Wednesday, Thursday and Friday morning.

A 10ml blood sample will also be collected on three occasions a few weeks after the experiment.

5. URINE SAMPLES

After ingesting the isotopes, urine should be collected according to the times stated on the bottles. These will be, for the first day: 0-3 hr; 3-6hr; 6-12hr and 12-24 hr after the experiment.

For the following 4 days (Tuesday to Friday) 24 hr urine samples will be collected approximately from 10.00am to 10.00am of the following day.

6. FAECAL SAMPLES

The complete faeces produced for each day from Monday to Friday, starting after the experiment, should be collected.

METHOD 15

COPPER STANDARD PREPARATION

Stock Standard is BDH Copper nitrate at 15.7 mmol/L.

Working Stock (prepared fresh monthly) at 1570 µmol/l. Weigh out 18g of water in a 25ml universal container (Sterilin) and add 2mls of stock copper standard.

Preparation of Working Standards (prepared fresh every 2 weeks)

Concentration copper µmol/l	**Weigh out H_2O (gms)**	**µls of conc. nitric added**	**Working Stock Standard added (µl)**
7.9	19.88	20	100
15.7	19.78	20	200
23.6	19.68	20	300
31.4	19.58	20	400

All pipette tips must be acid washed.

Standards are stored in a fridge at 4^0C, and remade every four weeks.

METHOD 16

STANDARD SERUM VALUES (Male)

Measured Values

		Ref. Interval	Method
Serum Cu	13.5 µmol/l +/-0.3	(11.5 - 23.5)	AA
Serum Cp	330 mg/l +/-10	(200 - 500)	Nephelometry
Total Protein	66g/l +/-1	(62 - 82)	Olympus Analyser
Albumin	42g/l +/-1	(37 - 53)	Olympus Analyser

Immunodiffusion Plate Results (average of two runs)

		Ring Diameter (mm)	Cp	% of Normal
1)	Std Male Serum	5.9	333mg/l	95
2)	Std after .45 µm filter	5.8	317mg/l	91
3)	Std after DEAE	5.6	301mg/l	86

Recovery from DEAE (small capacity column)

- Serum (2 mls) diluted 1:1 with running buffer
- Run at 2mls/min, collected as 3 x 2 ml fractions, measured by AAS flame:

Fraction (1) 5.9 µmol/l or 12 µmol/Cu
(2) 9.1 µmol/l or 18 µmol/Cu
(3) 1.9 µmol/l or 4 µmol/Cu
total 24 µmol/Cu

- 27 µmol/Cu in original serum therefore total Cu recovery = 89%.
- 6mls re-concentrated to 2mls (original serum volume) and measured for;

Total Protein = 9g/l (14% of 66g/l),
Ceruloplasmin = 0.3g/l
Albumin = 0g/l

- All the Albumin and 86% of total proteins removed.

MANUFACTURERS

Amicon Ltd
Upper Mill
Stonehouse
Gloucestershire
GL10 2BJ

Atlantic Antibodies-
Unipath Ltd
Bedford
MK41 0QG

BDH: McQuilkin & Co
21 Polmadie Avenue
Glasgow
G5 0BB

Becton Dickinson
Dublin
Ireland

Behring Diagnostics
50 Salisbury Road
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Middlesex
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Centrifichem
Baker Instruments
WhiteHall Lane
Surrey
TW20 9NW

Elga Ltd
Lane End
High Wycombe
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HP14 3JH

GBC Scientific Equipment PL
PO Box 1226
Dandenong 3175
Victoria
Australia

Gibco: Life Technologies Ltd
Trident House
Renfrew Road
Paisley PA3 4EF

Millipore SA
67120 Molsheim
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Nanosep Filtration Systems
Molly Millar's Lane
Wokingham
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Packard Instrument Co
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Pharmacia LKB
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Hertfordshire
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Philips Analytical
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Sebia: YSI Clandon Ltd
Lynchford Lane
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Hampshire GU14 6LT

Sherwood Medical
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Sigma Chemical Co Ltd
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Sterilin Ltd
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Middlesex
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8E Forge Parkway
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MA 02038
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VG Elemental: Fisons
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