DETERMINATION OF NON-CERULOPLASMIN-BOUND COPPER IN SERUM

A METHOD FOR THE DETERMINATION OF NON-CERULOPLASMIN-BOUND COPPER IN BLOOD SERUM AND SOME OF ITS APPLICATIONS

By

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SCOPE AND CONTENTS:

Methods have been developed to analyse for the non ceruloplasmin-bound copper and total copper in human blood serum accurately and precisely. The former was firstly isolated from the serum by using Chelex 100 resin. Both the non-ceruloplasmin-bound copper and the total copper were determined by neturon activation analysis, atomic absorption spectroscopy and a spectrophotometric method using zinc dibenzyldithiocarbamate as the chelating agent. Then the concentration of the ceruloplasmin-bound copper could be calculated by the difference. In vitro and in vivo radioisotopic labelling techniques were used in establishing the methods. Then they were applied to analyse the blood sera from some Caucasians, Chinese and a person with glomerulonephritis. The variations of the copper concentration as a function of time of one Chinese volunteer were also studied.

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DELINEATION OF THE PROBLEM*

The work reported in this thesis has three aims:

1. The development of a procedure for an analytically complete separation of non-ceruloplasmin-bound copper from ceruloplasmin-bound copper.
2. The comparison of three independent methods for the determination of very small amounts of copper.
3. The application of these analytical techniques to some specific physiological topics.

In any scientific work the intellectual conclusions are only as good as the data obtained in the laboratory. Although in many cases the accuracy and the precision of a given method are sufficient or better than required, in a substantial number of cases the analytical difficulties are often greater than appears at first glance. Today the demands in trace analysis are constantly increasing and a definite need exists to measure extremely small concentrations with comparatively high accuracy ($\leq 10\%$). This type of work requires scientists with great skills as well as a very critical mental attitude which allows the researcher to constantly search for possible sources of error.

Copper exists in human blood serum in two major

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* No references are cited in the text of this section as all the points raised here are discussed later in more detail.

Summary of Analyses of Standard Kale *

The Results of an Inter-Laboratory Comparison

Concentration of Copper ppm. (# in brackets = # of analyses)	Method of Determination
4.4(4), 3.6(2), 4.1(6), 4.6(15), 5.6(1), 6.5(6),	Neutron Activation Analysis
5.8(4), 5.4(5), 5.1(4), 6.0(1), 4.7(4), 6.3(4), 5.2(2), 6.3(7)	Atomic Absorption Spectroscopy
3.7(4), 4.7(5), 4.9(4), 4.3(4), 4.8(4), 4.8(4), 4.3(4), 4.6(3), 5.4(4), 4.3(4), 5.2(4), 4.4(4), 4.3(4), 6.5(4), 5.3(2), 9.5(5), 17(3)	Colorimetry
4.5(1), 5.5(4)	Polarography
6.4(4), 4.6(5)	Spectrometry

* from Bowen, H. J. M., Standard Materials and Intercomparisons, "Advances in Activation Analysis", Vol. 1 (Lenihan, J. M. A. and Thompson, S. J., ed.) Academic Press, London and New York, 1969.

fractions with the total concentration being ~1 ppm. The first fraction accounts for ~95%. This copper is incorporated in ceruloplasmin, a protein of molecular weight ~ 160,000. The second fraction (~5% = ~0.05 μg Cu/ml) contains most of the copper complexed by albumin and to a very small extent by free amino acids. The difference in binding strengths (either for thermodynamic or kinetic reasons) is substantial. Although this well-established fact immediately opens an avenue for the development of a separation technique, no entirely satisfactory procedure is currently available. It is of primary importance that reliable and accurate data (± 10% or better) can be obtained in order to draw conclusions of physiological significance. But even if the separation can be achieved the determination of the very small quantities of copper in the non-ceruloplasmin fraction poses another severe problem. In this connection an inspection of a compilation of analytical data published by Bowen is of interest (see opposite page [A-1]).

Two important conclusions can be drawn. Firstly, the scattering of the results within any one method is much larger as would be expected from an evaluation and summation of individual sources of error. For example, in neutron-activation analysis the lowest value is 3.6 ppm., the highest is 6.5 ppm. This corresponds to a discrepancy of almost 100%. Secondly, atomic absorption spectroscopy gives on the average high results. It should be realized that the concentration of copper in Bowen's kale is approximately 100 times higher than that in the non-ceruloplasmin fraction of blood serum.

It has always been the philosophy in analytical chemistry that the data are most convincing if two or more independent methods give the same result. Therefore, a comparison of methods for the determination of trace amounts of copper is essential.

There is not much immediate need for the development of a technique - however difficult it may be - if this method can not be applied to study a well-defined problem. It has been known for some time that the concentration of ceruloplasmin (and therefore the total copper concentration) in serum is hemostatically well controlled in healthy humans. However it is not known whether the same applies to non-ceruloplasmin copper. Another question is whether the concentrations of the two fractions of copper are the same for different population groups. (After this part of the present study was started a publication appeared showing that there are indeed substantial differences of this type.). Moreover another interesting question concerns the tightness of the hemostatic control within one individual. If the fluctuations in an individual are less than the distribution found within a group of similar individuals, significant conclusions can be drawn for clinical chemists and physicians. Imagine for a moment that the normal level of a metabolically important species in one particular person is low but within the so-called normal range. A pathological event occurs and the concentration of that species is pushed up by say 20%, but still within the normal range. Obviously the result of the standard test would mislead the physician. In fact there might well be a development in the future - based on the philosophy that prevention is better than treatment - which will implement a national health policy of yearly screening of every individual starting when a person is healthy.

During the course of this work, a paper appeared demonstrating a circadian cycle in ceruloplasmin levels but the non-ceruloplasmin copper was not measured. Moreover in many conditions - most importantly Wilson's disease, a malfunction of copper metabolism - the concentration of ceruloplasmin copper changes substantially. The same applies to women during the monthly cycle, pregnancy and women on "the pill". Again nobody knows if the same is true for non-ceruloplasmin copper. The effects of other drugs and other forms of treatment also deserve attention in this connection. Consequently an accurate and precise method for the determination of non-ceruloplasmin copper is definitely necessary to study the problems mentioned above.

INTRODUCTION

1.1. General.

Copper is one of the oldest metals known to man. Copper objects believed to date prior to 4000 B.C. have been found in Mesopotamia and Egypt. In spite of this, the presence of copper in living organims was not noticed until 160 years ago, when copper was found in plants[1,2] and later in animal tissues.[3] The findings of the copper containing respiratory pigment hemocyanin in cold-blooded animals[4], and the turacin, a copper containing pigment in the feathers of certain birds[5] gave an indication that copper was an important body constituent. Hart _et al._[6,7] later showed that copper was an essential nutrient for the rat and other animal species. Copper has now been recognized to be an essential element in human metabolism. It is an integral part of a number of proteins. Many of these copper proteins are enzymes, and the copper is a part of their active group; for example, cytochrome C oxidase[8-11], while others have no known enzyme activity, for example, cytocuprein.[12-16] Vallee[17] designated these proteins a special class of metal-protein complexes and established them as metallo-proteins. A good monograph "Metallo-proteins" written by Vallee and Wacher[18] has been published recently.

The total copper content of the normal human adult is estimated to be 50-120 mg , based on a quantitative emission spectrographic analysis in 29 human tissues by Tipton and Cook.[19-20]

The liver, brain, heart muscle and kidney have the highest concentration of copper, in decreasing order.

The blood level of copper is about 100μg /100 ml (1ppm.).[21] The serum and erythrocytes contain similar concentrations of copper. This copper does not exist in free ionic form under physiological condition. About 90-95% of the copper in serum is bound to a blue protein called ceruloplasmin[22-24], with a molecular weight of ~160,000. This protein is an α_2-globulin and contains eight atoms of copper per molecule. In addition to being a metallo-protein, it is also a glycoprotein containing 7% carbohydrate: hexose, hexosamine and neuraminic acid.[25] Of the eight atoms of copper in ceruloplasmin, four are in the cupric state, while the other four are in the cuprous state.[26-29] The blue color and the enzyme activity towards *p*-phenylenediamine or its dimethyl derivative are dependent on the presence of the cupric ion.[28] It has been shown that exchange between ceruloplasmin bound and ionic copper does not occur under physiological conditions. Copper is only incorporated into the protein when it is synthesized in the liver and the copper is later released only during the decay of the protein molecule.[30-35]

Of the remaining small fraction of serum copper, most of it is attached to serum albumin. It is called "non-ceruloplasmin bound copper" or "direct-reacting copper".[30] There are indications that the amount of this fraction of serum copper varies in physiological and especially in pathological conditions, of which Wilson's disease is known to be a disturbance of copper metabolism.[20]

Bearn and Kunkel[36,37] found that following the injection or intravenous administration of ^{64}Cu, the ^{64}Cu is bound exclusively to serum albumin during the early stages. It is suggested that the portion of serum copper which serves as an intermediate transport form of copper and is in equilibrium with tissue copper is not the large compartment of ceruloplasmin, but the 5-10% of copper loosely bound to albumin.[30] Moreover, in addition to these two fractions, Neuman and Sass-Kortsak[38,39] Sarkar and Kruck[40] have shown that there is a third small fraction of copper in human serum bound to amino acids. This fraction is only a few percent of the non-ceruloplasmin bound copper. They suggested that copper may be present in serum in the form of mixed amino acid copper complexes consisting of one atom of copper and two different amino acids. Under normal conditions, histidine is the amino acids primarily involved in the formation of mixed amino acid copper complexes in serum. Threonine, glutamine or asparagine are the other amino acids involved. This amino acid bound fraction of copper is in equilibrium with albumin bound copper. Although this fraction is rather small compared even to the albumin bound fraction it may have a physiological role in the biological transport of copper because of its small molecular size.[39]

The aim of the work presented in this thesis is to find methods to analyse for the non-ceruloplasmin bound copper and the total copper in human blood serum accurately. This has been achieved by isolating the non-ceruloplasmin bound copper from the serum by Chelex 100 resin. This copper was then determined by neutron activation analysis, atomic absorption spectroscopy or spectrophotometric analysis using zinc dibenzyldithiocarbamate as a chelating agent. Radio-isotopic labelling techniques were used together with gel-chromatography in establishing the methods. These methods were then applied to analyse for the non-ceruloplasmin bound copper and total copper in the blood sera of some Caucasians and Chinese. The variations of the copper concentrations as a function of time of one Chinese subject were also studied.

1.2. Determination of Total Copper in Blood Serum.

Many methods are available to determine trace amounts of copper in blood serum and other biological tissues. Neutron activation analysis, atomic absorption spectroscopy and spectrophotometric methods are widely used. These will be discussed more fully in the following sections. Other methods such as emission spectrography,[19,41-47] X-ray fluorescence,[48] polarography,[49] and recently kinetic methods[50-52] have also been used.

1.2.1. Neutron Activation Analysis (NAA).

Neutron activation analysis originated with the classical papers of Hevesy and Levi[53] in 1936 and of Seaborg and Livingood[54] in 1938. It has been proven to be an important method for the determination of trace elements in the past fifteen years. It has been used extensively in the biological, earth, physical, environmental and forensic sciences. This remarkable growth in application can be attributed not only to the acceptance of neutron activation analysis as a successful method for trace element analysis and the increasing availability of nuclear reactors, but also to the development of sophisticated radiochemical separation methods and radiation detection instruments.

The basic principle of the method is that stable isotopes, when irradiated by neutrons, can undergo a nuclear transformation to produce radioactive nuclides. By measuring the radiation emitted by the radioactive nuclides, the desired elements can be determined qualitatively and quantitatively with or without separations. The induced activity at the end of irradiation (A, disintegration sec^{-1}) depends on the number of target nuclides (N), the neutron flux (ϕ, neutrons cm^{-2} sec^{-1}), the neutron cross section (σ, $10^{-24} cm^2$), the irradiation time (t), and the half-life of the product nuclide ($T_{1/2}$). The relationship is given by the radioactivity production equation, which commonly is written in the form:

$$A = N\phi\sigma(1-e^{-0.693t/T_{1/2}})$$

A more detailed discussion on the theory and practice is available from the books recently written by Kruger[55], De Soete *et al.*[56] and Hoste *et al.*[57]

Neutron activation analysis is presently one of the most promising methods for the determination of copper on the ultramicroscale. There are two naturally occurring stable copper isotopes, ^{63}Cu and ^{65}Cu. Exposure to a high flux of thermal neutrons would convert these to radioactive ^{64}Cu and ^{66}Cu respectively. Some of their properties are shown in

Table I.

The use of ^{64}Cu for detection is preferrable over ^{66}Cu. This is due to the larger percentage abundance and larger thermal neutron cross-section of its parent isotope, longer half-life of the induced ^{64}Cu, and higher intensity of the gamma-ray radiation. Consequently, ^{64}Cu is easier to be measured accurately and precisely.

Copper in serum, plasma or whole blood has been analyzed by neutron activation analysis for some years.[59-91] Nondestructive instrumental analysis is impossible due to the large amount of sodium present.[176] ^{24}Na ($t_{1/2}$ - 15 hrs) is produced during activation. The compton radiation from the large 1.37 Mev. photopeak of ^{24}Na completely obscures the relativelysmall 0.511 Mev. annihilation radiation of ^{64}Cu. Thus the copper must be separated from the sodium, either preceding the neutron activation or, more conveniently, after the activation step. In the latter case, stable copper may be added as a carrier. This enables the separation to be carried out on a macroscale instead of a microscale. Furthermore, contamination with inactive copper at this stage does not affect the result and total recovery is not required if the chemical yield of the separation is also determined.

Kaiser and Meinke[68] developed a rapid activation analysis for trace copper using the 5.1 minute ^{66}Cu. Copper in the sample was extracted by cupferron into carbon tetrachloride and finally precipitated as CuS for the measurement of activity. The sensitivity of this method

TABLE I

Nuclear Properties of Copper[58]

Stable isotopes	^{63}Cu	^{65}Cu
% of abundance	69.1	30.9
Thermal neutron cross section (barns)	4.5	2.3
Induced radioactive nuclides by (n, γ) reaction	^{64}Cu	^{66}Cu
Half-life of induced nuclides	12.8 hrs.	5.1 mins.
Types of decay	EC (43%) β^- (38%) β^+ (19%)	β^-
Major radiations (Mev.)	β^-0.573 β^+0.656 γ0.511 (38% $\gamma\pm$) 1.34 (0.5%)	β^-2.63 γ1.039 (9%)

was only 3×10^{-7} g copper. Bowen[59] used the 12.8 hours ^{64}Cu together with a chemical method which involved repeated precipitations with thiocyanate and finally with salicylaldoxime. He obtained a sensitivity of 5×10^{-10} g copper. Other separation methods, such as solvent extraction,[63,81] ion exchange chromatography[73,82] and dialysis before activation[72] have also been suggested.

1.2.2. Atomic Absorption Spectroscopy (AAS).

In 1955, Walsh[92] published the first paper on atomic absorption spectroscopy as an analytical method. Nowadays, atomic absorption spectroscopy has reached the status of one of our major analytical methods. More than sixty elements can be determined routinely with this method. It is used extensively in clinical, biomedical, agricultural, geochemical, metallurgical and pollution studies.

Basically, atomic absorption spectroscopy involves aspiration of the sample solution into a flame where the metal ions are converted into atomic vapor state. Most of this atomic vapor exists in the ground electronic state and can therefore absorb resonant radiation of an appropriate wavelength. A hollow cathode lamp is usually used as the source. This is a sharp line source consisting of a cathode made of the particular element in question. The lines characteristic of the element are emitted from the cathode and pass through the flame where a fraction of it is absorbed by the atomic vapor. The absorption obeys Lambert-Beer's Law to a certain extent. Since only the test element can absorb the radiation, the method becomes very specific. In addition, because most of the atomic vapor exists in the ground state, atomic absorption is sensitive for a large number of elements. This is in constrast to flame photometry where the light emitted from the small amount of excited atomic vapor is measured. More information about the theory and application of atomic absorption spectroscopy is available through the monographs recently published.[93-95]

Allan[96] was the first scientist to study the determination of copper by atomic absorption spectroscopy in a systematic manner. The copper spectrum provides several lines that are analytically useful. This is shown in Table II.

Allan[96] recommended that the absorption line at 3247.5 Å be used for the determination of copper. The sensitivity was about 0.1 μg/ml/1% for an aqueous solution of copper in an air-coal gas flame. Willis[98] recommended the 2492.1 Å line for copper when it was determined as a major constituent and found that the sensitivity was about 4.2 μg / ml / 1%. The sensitivity may be increased several fold by extracting the copper into an organic solvent. Ammonium pyrrolidine dithiocarbamate is usually used as a complexing reagent to extract copper into ethyl acetate or methyl isobutyl ketone.[96,99,100]

Atomic absorption spectroscopy has been studied to determine copper in blood serum, plasma and whole blood.[101-121] The advantages and disadvantages of these proposed methods

TABLE II

Relative Sensitivity of Various Copper Lines in Absorption[96]

Wavelength (Å)	Sensitivity (μg/ml/1%)
2225.7	2
2244.3	22
2441.6	55
2492.1	9
3247.5	0.1
3274.0	0.2

and the one suggested in these studies will be discussed in section 3.6.

1.2.3. Spectrophotometry

The wide use of spectrophotometric methods in chemical analyses is based on the possibility of converting the constituent to be determined into a substance which in solution (or suspension) is strongly colored. Such a solution shows differential absorption of light of different wavelengths. When the percentage of light transmitted by the solution is plotted against the wavelength, a transmission curve is obtained, which usually shows one or two pronounced maxima or minima, but which sometimes may be more complex. Alternatively, the percentage of light absorbed may be plotted against the wavelength, and an absorption curve is then obtained.

When dilute solutions are involved, the following relation is found to hold closely:

$$A = \log I_o/I = abc$$

in which A - Absorbance

I_o - Intensity of the incident monochromatic light

I - Intensity of the transmitted light

a - Absorptivity

b - Light path length

c - Concentration of the colored substance

This relation is known as the Lambert-Beer Law and is the fundamental law of spectrophotometry.

The absorptivity (or molar absorptivity, ε, if concentration is expressed in mole/litre instead of g/l) can be greatly increased by liquid-liquid solvent extraction of the ion-association complexes or the metal chelates into an organic solvent. The introduction of the very versatile organic reagent "dithizone" by Fischer[123] in 1925 has opened a new era in using spectrophotometric methods in chemical analyses. Since then, many chelating reagents have been found and many spectrophotometric methods have been established to analyze for most of the elements in the periodic table. A comprehensive study of these methods may be referred to the monographs by Sandell,[124] Morrision and Freiser,[125] and Stary.[126]

There are a great number of color reagents for spectrophotometric determination of copper in various substances. Table III gives a list of the reagents most commonly used. Several new colorimetric reagents were also investigated during the past few years.[131-160]

1.3. Determination of Non-ceruloplasmin Bound Copper in Blood Serum.

As mentioned in section 1.1., copper in blood serum is predominantly bound to the protein ceruloplasmin. Only a small fraction (about 5%) is bound to albumin and amino acids. This small percentage makes it difficult to determine the non-ceruloplasmin bound copper accurately. One approach is to determine both the total copper and the amount of ceruloplasmin in serum. Then the non-ceruloplasmin bound

TABLE III[21]

Spectrophotometric Reagents for Copper

Reagents	Solvent	Wavelength (nm)	Molar Absorptivity	References
Diethyldithiocarbamate	water	440	9,600	127, 128
	amyl alcohol	440	12,700	128 - 130
	chloroform	435	14,700	131
Dibenzyldithiocarbamate	CCl_4	435	16,200	131, 133, 134
Diphenylthiocarbazone (dithizone)	CCl_4	508	24,600	124, 126, 135
2,2'-Biquinoline (cuproine)	amyl alcohol	540	5,900	128, 136-139
2,9-Dimethyl-1, 10-phenanthroline (neocuproine)	$CHCl_3$-EtOH	454	8,000	128, 140, 141
2,9-Dimethyl-4, 7-diphenyl-1, 10-phenanthroline (bathocuproine)	n-Hexanol	480	14,200	131, 142, 143
Biscyclohexanoneoxalyldihydrazone (cuprizone)	water	600	16,000	128, 144, 145
Oxalydihydrazide + acetaldehyde	water	542	29,500	146 - 148
1, 5-Diphenylcarbohydrazide	water	495	158,000	149
	Benzene	540	55,000	150

copper is obtained by difference. This would not be accurate due to the uncertainty in the molecular weight of the ceruloplasmin and the percentage of copper in the protein. Moreover, the difference would give only an approximate value for the non-ceruloplasmin bound copper as a large error is introduced by the substraction of two large numbers. The second approach is to determine the non-ceruloplasmin bound copper directly. This is a more logical approach. Moreover, the non-ceruloplasmin bound copper offers a better means of evaluating the copper status of an individual than total serum copper because it reveals fast changes in copper supply in contrast to ceruloplasmin, or liver copper content. The determination of the latter is insufficient to show any changes within a few weeks when the values start to deviate from the normal range. The determination of the non-ceruloplasmin bound cooper would allow judgement on the absorption from the gut within a few hours.

Several methods were suggested to determine the non-ceruloplasmin bound copper directly, but none of them is satisfactory. Gubler <u>et al.</u>[38] proposed a spectrophotometric method. The blood plasma was diluted with water. Sodium pyrophosphate was used to buffer the solution and sodium diethyldithiocarbamate was then added. The absorbance of the developing faintly yellow color of the copper diethyldithiocarbamate complex was measured at 440 nm. Correction was applied for the color of the plasma by a preliminary reading, before the addition of the color reagent and this correction was also to be adjusted for the dilution caused by the volume of the diethyldithiocarbamate solution. Due to the low concentration of non-ceruloplasmin bound copper, the absorbance reading would be very low. Assuming the concentration of non-ceruloplasmin bound copper is 0.05 μg/ml, the absorbance would be only 0.002. Actually the blood serum or plasma was not colorless, but somewhat yellow. Thus a great error might be introduced for the correction of the color of the plasma. Consequently, the method is not suitable for an accurate analysis for non-ceruloplasmin bound copper. A more detailed discussion of this method was given by Binnerts and Boom.[162]

Brown[161] suggested another spectrophotmetric method. The deep pink-colored copper complex with bis-acetaldehyde-oxalydihydrazone was used to determine the copper content. The disadvantage is that the pH control for the complex formation was critical. Maximum color development was observed only within pH range of 8.4 - 9.1. The low absorbance of the sample solution would also cause uncertainty in measurement. Another critical point is that the behaviour of the non-ceruloplasmin bound copper and ceruloplasmin bound copper with the chelating reagent were not studied. Furthermore, no experimental result was shown. This makes it impossible to estimate the accuracy and precision of the method.

Blomfield and MacMahon[163] suggested a method by atomic absorption spectroscopy. The non-ceruloplasmin bound copper was chelated by ammonium pyrrolidine dithiocarbamate and extracted into n-butyl acetate. The organic extract was aspirated into the flame of the atomic absorption spectrophotometer and the absorbance was measured. This suffers from the same disadvantages as in the method of Gubler et al.[30]. Low absorbance readings are obtained. Furthermore it is found in these studies that the degree of extraction of the copper complex into the organic solvent is not reproducible. It varies from 90 to 98%, from an experiment by adding ^{64}Cu to serum as a tracer. This may explain partly the poor precision of the method. In their experiments, duplicate determinations on ten different days with aliquots from a single blood sample were performed. They obtained a result of 5.9 ± 1.1 µg/100 ml. The coefficient variation of reproducibility was 18.6%.

A similar method by atomic absorption spectroscopy was suggested by Binnerts and Boom.[162] They used diethyldithiocarbamate as the chelating reagent. The copper complex was extracted into methyl isobutyl ketone and the organic layer was then sprayed into the flame for the measurement of absorbance. To account for the incomplete extraction, ^{64}Cu was added before extraction. In this case, the recovery can be corrected by simple isotope dilution. The use of ^{64}Cu introduces new problems. Firstly, unless high specific activity ^{64}Cu is used, the amount of ^{64}Cu added to the sample would be appreciable. In their experiments, 0.4 to 2 µg copper (depending on the age of the irradiated copper) was added to 10 ml cow plasma which contained ~1-2 μg non-ceruloplasmin bound cooper. Thus a sizeable proportion of the absorbance signal would come from the added radioactive copper. The situation is even worse with human serum, in which the concentration of non-ceruloplasmin bound copper is less than that in cows. Thus the absorbance is contributed mainly from the added copper tracer instead of from the non-ceruloplasmin bound copper which is the aim of the experiment. Moreover, the large sample size, 10 ml of serum, corresponding to 20 ml of blood, used in one measurement is quite impractical. Secondly, frequent preparation of ^{64}Cu is necessary due to the short half-life of the isotope (12.8 hr.). Furthermore, the author did not give results for multiple analyses, so that the precision of the method could not be evaluated. But based on the reasons mentioned above, it is quite doubtful that the method would provide a means for accurate and precise analyses.

As can be seen, current methods available for determination of non-ceruloplasmin bound copper are not entirely satisfactory. In order to have an accurate study on the levels of this copper in normal and pathological sera, a new independent method has been developed in these studies.

EXPERIMENTAL EQUIPMENT AND PROCEDURES

2.1. Materials and Reagents*

The chelating resin Chelex 100[160], 200-400 mesh size, was obtained from the Bio-Rad Laboratories (Richmond, California, U. S. A.). Because of its high affinity for transition-metal ions, the commerically available resin contains some copper and has to be purified. This was done by extensive washing with a high purity 1M HCl solution which was prepared from B. D. H. Aristar grade hydrochloric acid and deionised distilled water. It was then washed extensively and finally suspended in a 0.15M NH_4OAc solution of pH 7.4, which was prepared from ammonia solution and acetic acid (both B. D. H. Aristar grade solution) and further purified by passage over a short Chelex 100 resin column (0.6 x 3 cm).

The columns used for separation of non-ceruloplasmin bound copper from ceruloplasmin bound copper were made from quartz tubing. They were soaked in aqua regia for twenty-four hours in order to leach out metal ions in the vicinity of the surface. After thorough washing with deionised distilled water, the Chelex 100 resin was allowed to settle in the column by gravity with some quartz wool plugged at the end. It was then washed with the high purity ammonium acetate solution.

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* In all work, standard analytical grade reagents were used without further purification unless specified.

Bio-Gel P-150 (#37473) was also obtained from Bio-Rad Laboratories. The gel is a cross-linked copolymer of acrylamide and methylene bisacrylamide, produced in the form of spherical beads that can be hydrated readily in water or salt solutions.

The copper standard used in neutron activation analysis was a known quantity of an aluminum alloy, in a sheet form, which contained 0.123% copper, and was obtained from the Aluminum Company of Canada.

The copper standard solutions for atomic absorption spectroscopy and spectrophotometric analysis were prepared by dissolving electrolytic grade copper wire and diluting with deionised distilled water.

Zinc dibenzyldithiocarbamate was obtained from Eastman Kodak Company (New York, U. S. A.). This was recrystalized by adding 95% v/v aqueous ethanol to a concentrated solution of the salt in carbon tetrachloride to the stage of incipient precipitation. It was then filtered, washed and dried. The product had a melting point of 187-188°C.

Ammonium pyrrolidinedithiocarbamate, methyl isobutyl ketone and trichloroacetic acid were obtained from Fisher Scientific Co. Benzene was obtained from Mallinckrodt Chemical Works Limited.

The polyproylene test tube (12 x 75 mm) used in atomic absorption spectroscopy and spectrophotometric analysis was obtained from Falcon Plastics (Oxnard, California, U. S. A.).

The 5 ml polyethylene bottle used in neutron activation analysis was obtained from Canus Equipment Ltd. (Ottawa, Canada). Both of these were rinsed with deionised distilled water and dried before use.

Both the plastic syringe (20 ml) and hypodermic needle (20 G 1.5) were obtained from Becton, Dickinson & Co., Canada.

Carrier-free ^{64}Cu was produced by the (n, p) reaction of 69 grade zinc pellets (Consolidated Mining and Smelting Company of Canada, Ltd.) in the McMaster University Nuclear Reactor. A zinc pellet (~300 mg) was irradiated in a quartz capsule for twenty-four hours in a cadmium envelope. After cooling for another twelve hours, the zinc pellet was rinsed briefly with 2M HCl. Then, it was dissolved in 3 ml concentrated HCl, in a teflon beaker. A few drops of conc. HNO_3 might be added to speed up the dissolution. The solution was evaporated to dryness under an infrared lamp. The residue was dissolved in 2 ml of 0.01M HCl. It was added to the top of a Chelex 100 resin column (4 x 0.6 cm) which was previously equilibrated with 0.01M HCl. The column was then washed with 20 ml of 0.01M HCl. Zinc was found not be adsorbed on the column under these conditions. Copper was then eluted out of the column with 1M HCl into a teflon beaker. The solution was evaporated to dryness. At this stage, the gamma-ray spectrum was pure ^{64}Cu and ^{67}Cu (produced by the (n, p) reaction of ^{64}Zn and ^{67}Zn), with the former activity about 0.5 mCi. The specific activity will be determined by the copper content of the zinc metal. The high purity zinc contains 0.1 - 0.2 ppm Cu. Thus the ^{64}Cu will have a specific activity of ∿8 mCi ^{64}Cu/μg Cu. This ^{64}Cu can be taken up by a small amount of physiological saline solution for *in vivo* experiments. Different concentrations of hydrochloric acid used in the procedure were prepared by diluting Aristar grade hydrochloric acid with deionized distilled water. Another separating procedure to isolate out the ^{64}Cu was suggested by Fritze.[164]

2.2. Instrumentation.

During gel-chromatographic fractionations, the eluate was collected on a L.K.B. Ultra-Rac fraction collector. The protein content of the eluate was monitored meanwhile with an L.K.B. Uvicored Spectrophotometer at a wavelength of 254 nm.

Irradiations for neutron activation analysis and production of radioisotopes were carried out in the McMaster University Nuclear Reactor with a thermal neutron flux of ∿2 x 10^{13}n cm^{-2} sec^{-1} and a cadmium ratio of ∿20. The flux was increased to ∿4 x 10^{13}n cm^{-2} sec^{-1} in the later stages of the study. Short term irradiations (less than twenty minutes) were done using the pneumatic rabbit system.

Radioactivity measurement was carried out by gamma-ray spectroscopy using the combination of the following detectors and analysers:

1. A co-axial Ge(Li) solid state detector (active volume of $40cm^3$) with an Ortec 118A preamplifier, Hewlett-Packard linear amplifier, and Nuclear Data 1024 channel analyser. The later was changed to another Nuclear Data 2048 channel analyser together with two Canberra stabilization pulsers and two Canberra digital stabilizers for zero and gain stabilization.

2. A 3 x 3 inch NaI(Tl) crystal with a Victoreen linear amplifier and a Nuclear Data 256 channel analyser.

3. A 3 x 3 inch well-type NaI(Tl) crystal with a Victoreen linear amplifier, a Canberra single channel analyser and a Nuclear-Chicago scaler-timer.

In the atomic absorption spectroscopy, the absorbance was measured with the Jarrell Ash 800 Atomic Absorption Spectrophotometer with a 10 cm path length premix burner and an air-acetylene flame.

As to the spectrophotometric analysis, a Cary 14 spectrophotometer was used. Absorbance was measured through a 10 mm cylindrical quartz curvette with a volume of about 0.8 ml. (Hellma Ltd.).

2.3. Sample Preparation

In all *in vitro* radioactive tracer experiments, human blood serum and pig plasma were used, while rat blood serum was used for *in vivo* studies. Blood samples were withdrawn using clean sterilized stainless steel needles and plastic syringes. Human blood samples were obtained from volunteers through a vein in the arm in the morning before breakfast. Rat blood samples were obtained by cardiac puncture. They were stored in quartz centrifuge tubes which had been soaked in aqua regia for twenty-four hours and thoroughly rinsed with deionised distilled water.

Serum was prepared from human and rat blood by allowing the blood to coaguate at 37°C for three hours. It was then centrifuged at low speed for 25 minutes. Only non-hemolysed serum was used in the studies.

2.4. Gel Chromatography.

The glass column used was cleaned with aqua regia and then thoroughly washed with dionised distilled water. It was dried and then siliconised with one per cent solution of dimethyl dichlorosilane in benzene (Bio-Rad Laboratories).

The dry gel, Bio-Rad P-150, was swollen for twenty-four hours in pure 0.15M ammonium acetate buffer (pH 7.4) containing 0.01M sodium cyanide. Gel columns were then packed by allowing the gel slurry to settle down in the column with the end plugged with white beach sand and cotton wool. The column was then more firmly packed under a low pressure head to maintain a slow flow rate. The column was further washed thoroughly with pure 0.15M ammonium acetate buffer. Then, the pressure head was increased until the desired permanent flow rate was attained.

In general, about 1 ml serum was fractionated on the column. Sucrose was dissolved in the sample solution to form a solution somewhat denser than the eluting buffer.

This was then gently layered on the flat surface of the column under the buffer already there. Care in this operation precluded any significant mixing of these two phases. The sample was then allowed to soak in about 1 cm below the surface. The reservoir was replaced on the top of the column and the pressure head was adjusted to the desired flow rate. A short column of Chelex 100 resin (pH 7.4) was incorporated at the base of the buffer reservoir to maintain a low metal ion concentration and to reduce the risk of contamination by extraneous metal ions. The eluate was monitored through a L.K.B. Uvicord Spectrophotometer to give a protein elution curve. The activity was later counted with the 3 x 3 inch well-type NaI(Tl) crystal together with a Canberra single channel analyser and a Nuclear-Chicago scaler-timer. Total elution time was about twenty hours.

2.5. Separation of Non-ceruloplasmin Bound Copper from Ceruloplasmin Bound Copper in Blood Serum.

The chelating Chelex 100 resin is known to bind copper from a solution of pH∼7 very strongly. A Chelex 100 resin column (200-400 mesh size) of size 0.6 x 26 cm was prepared from the purified resin as mentioned in section 2.1. It was equilibrated with 0.15M ammonium acetate solution of pH 7.4 which was prepared from B.D.H. Aristar grade ammonia and acetic acid.

A serum sample of two to three ml was applied to the top of the resin bed and allowed to soak in. The column was then washed with 30 ml of high purity ammonium acetate solution through a reservoir. A short column (0.6 x 3 cm) of Chelex 100 resin was inserted between the reservoir and the main separating column for further purification and to reduce the risk of contamination by extraneous metal ions. The eluate and wash solution containing among all the other proteins, the ceruloplasmin, were rejected. The copper bound originally to albumin and amino acids was adsorbed on the column under these conditions. This was established through the <u>in vitro</u> and <u>in vivo</u> experiments discussed in section 3.1. and 3.2. The adsorbed copper could easily be stripped off the column with high purity 1M HCl prepared from diluting B.D.H. Aristar grade concentrated hydrochloric acid with deionised distilled water. As the acid passes through the column, the appearance of the resin changed towards a slightly darker color. Therefore the collection of the desorbed copper was only begun when the acid front was ~0.5 cm from the end of the column. From this moment on, 3.5 ml solution was collected which contained all the copper originally adsorbed on the column. This might be analyzed for copper by neutron activation analysis, atomic absorption spectroscopy or spectrophotometric methods. The blank for the separation was obtained by running another Chelex 100 resin column in parallel with the sample column but omitting the addition of the serum sample.

2.6. Determination of Non-ceruloplasmin Bound Copper in Blood Serum.

After separation of the non-ceruloplasmin bound copper from ceruloplasmin bound copper, the former might be determined by one of the three methods described below.

2.6.1. Neutron Activation Analysis.

The 3.5 ml of 1M HCl eluate from the Chelex 100 resin column was collected in a 5 ml polyethylene bottle. After freeze-drying, the bottle was heat-sealed and irradiated for two hours together with a copper standard, (a known quantity of an alumium alloy containing 0.123% copper) in the McMaster University Nuclear Reactor at a flux of 2×10^{13} n cm^{-2} sec^{-1}.

After irradiation, the sample was allowed to cool for twelve hours before chemical separation was carried out. The active material in the irradiated capsule was leached out quantitatively into a 30 ml beaker with a hot concentrated nitric acid solution in the presence of 2 mg each of copper and sodium carrier. The latter was used as a hold back carrier. The solution was evaporated to near dryness, diluted with water and then neutralized with ammonia until the slightly blue color of the copper-ammonia complex appeared. The final volume was about 4 ml. This was added to the top of a short Chelex 100 resin column (0.6 x 3 cm , 100-200 mesh size). The beaker was rinsed with four fractions of 5 ml 0.15M NH_4OAc solution and the solution was added to the column. The adsorbed copper (a blue band on the top portion of the resin column) was then eluted with 1M HCl and was collected in a 10 ml volumetric flask in the same way as in the separation procedure mentioned above.

The copper standard was dissolved in 5 ml of 4M HCl and four drops of nitric acid. It was diluted to 100 ml in a volumetric flask. An appropriate amount (to give approximately the same count rate as the sample) was transferred to a 10 ml volumetric flask and again diluted to the mark. The induced ^{64}Cu activity was measured using a high resolution Ge(Li) detector in conjunction with a multi-channel analyser. The amount of copper in the sample can then be calculated by comparison of the activities in the sample and standard.

2.6.2. Atomic Absorption Spectroscopy.

For the atomic absorption analysis, the copper eluted by the 1M HCl was collected in a 6 ml polypropylene centrifuge tube. After addition of 100 μl of 1% aqueous solution of ammonium pyrrolidine dithiocarbamate and 1.50 ml methyl isobutyl ketone, the closed tube was manually shaken for three minutes and then centrifuged to achieve complete phase separation. Eppendorf microlitre pipettes of appropriate volumes were used for measuring the volumes of the reagent solutions. The upper organic layer was then aspirated into the acetylene-air flame of the Jarrell Ash 800 Atomic Absorption Spectrophotometer with a 10 cm path length premix burner. The absorbance was measured at a wavelength of 3247.5 Å. The calibration curve was obtained in the same way using standard solutions containing 0.102 μg , 0.255 μg , 0.509 μg , 0.764 μg , and 1.020 μg copper in 1M HCl medium. The calibration curve is shown in Figure VIII.

2.6.3. Spectrophotometric Method.

Zinc dibenzylditiocarbamate was chosen as the chelating agent in these studies. Exactly 1.00 ml of a 0.06% solution of this reagent in benzene was used for the extraction. It was added to the 6 ml polypropylene centrifuge tube containing the copper solution eluted from the Chelex 100 resin column. The closed tube was shaken for three minutes and then it was centrifuged to achieve complete phase separation. The upper benzene layer was then transferred into a 10 mm cuvette (volume ∿0.8 ml) with a clean disposable pipette. The absorbance at 438 nm was measured in the Cary 14 Spectrophotometer. The calibration curve was obtained from similarly extracted standard solutions containing different amounts of copper. The calibration curve is shown in Figure XIV.

2.7. Determination of Total Copper in Blood Serum.

The total serum copper was also determined using the three methods described above. For the neutron activation analysis, a known amount of serum (0.5 - 1 ml) was freeze-dried. After neutron irradiation, the sample was digested in a long-neck Kjeldahl flask with a mixture of nitric acid and sulfuric acid in the presence of copper and sodium carrier. After a colorless, clear solution was obtained, the solution was heated vigorously until white fumes of sulfuric acid appeared. It was cooled down and ammonia was used to neutralize the solution. The ^{64}Cu activity was isolated and measured in the same way as in the determination of non-ceruloplasmin bound copper.

In the atomic absorption and spectrophotometric analyses, serum was put in a 6 ml polypropylene centrifuge tube with a cap. The proteins of the serum were precipitated by 1.00 ml of 3N HCl solution (prepared by diluting B.D.H. Aristar grade HCl with deionised distilled water) and 1.00 ml of 20% trichloroacetic acid. The tube was centrifuged for 10 minutes. The supernatant which contained copper was quantitatively transferred into another centrifuge tube. The precipitate was washed with 1.00 ml deionised distilled water. The tube was centrifuged again and the supernatant was added to the previous one. Then the copper content in this combined solution was determined by either atomic absorption spectroscopy or the spectrophotometric method as described in the previous sections. As to the standards, the same amount of hydrochloric acid and trichloroacetic acid were added to the aqueous standard solutions before extraction.

RESULTS AND DISCUSSION

3.1. In Vitro Addition of Copper-64 to Human Serum.

The chelating Chelex 100 resin[163] was known to bind copper ion from a solution of pH$\sim$7 very strongly. Experiments were carried out to try to separate ceruloplasmin bound copper and the non-ceruloplasmin bound copper with the resin. It was hoped that the copper bound to ceruloplasmin would pass through the resin directly without being adsorbed, whereas the copper bound to albumin and amino acids would be removed and adsorbed on the resin. The adsorbed copper could be removed later by treating the resin with hydrochloric acid.

To test this idea, fresh human serum was spiked with carrier-free ^{64}Cu and fractionated on a Bio-Gel P-150 column (1.6 x 35 cm). The ^{64}Cu activity was related to the protein chromatogram as shown in Figure I. The three peaks in the protein distribution curve (solid line) have been assigned by Porath[165] as follows: the first (left hand) peak contains the high molecular weight serum components (α- and β-lipro-proteins and α- and β-globulins), the centre peak contains 7S γ-globulin and the third peak is albumin. It can be seen from Figure I that all the ^{64}Cu activity occurred in one peak and the peak coincided with the albumin peak in the protein fractionation curve.

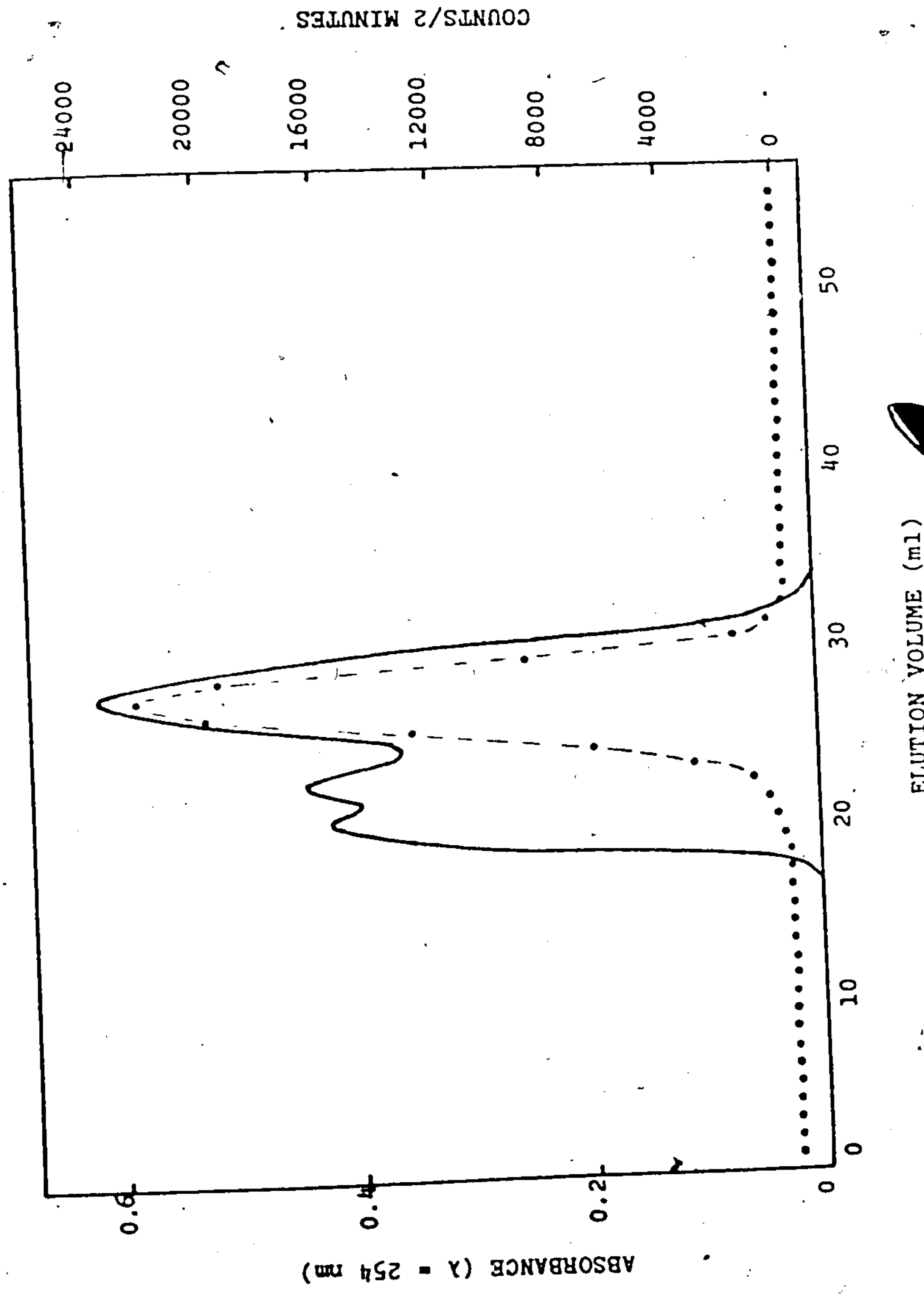
FIGURE I.

Human Blood Serum Spiked with ^{64}Cu and Chromatographed on a Bio-Gel P-150 Column (1.6 x 35 cm).

Sample Size: 0.5 ml.

Protein Distribution Curve - Solid Line

^{64}Cu Activity Curve - Dot Line


COUNTS/2 MINUTES
24000
20000
16000
12000
8000
4000
0
ABSORBANCE (λ = 254 nm)
0.6
0.4
0.2
0
ELUTION VOLUME (ml)
0
10
20
30
40
50

For the separation with Chelex 100 resin, columns of different sizes were prepared. After equilibration with a 0.15M high purity ammonium acetate solution of pH 7.4, 2 ml of the ^{64}Cu spiked serum sample were added to each column. The column was then washed with ten resin-bed volumes of ammonium acetate solution. The adsorbed copper was later removed by washing the resin with 1N HCl solution. As can be seen from Table IVa, the degree of retention of ^{64}Cu on the columns depends very much on the mesh sizes of the resin, the length and the flow rate. As any further increase in length appeared impractical, a column size of 0.6 x 26 cm was arbitrarily chosen, and the flow rate was reduced by inserting a thin polyethylene tube at the outlet of the column. Under these conditions, the retention of ^{64}Cu was quantitative (>98%). Further experiments showed that the retention was quantitative and reproducible with resin of mesh size 200-400, a length of column between 25 to 30 cm, and a flow rate 2.0 - 2.5 ml/hr. It was also established later that the serum sample used can be up to 4.5 g without overloading the column. This is shown in Table IVb.

Furthermore, as the hydrochloric acid used to elute the adsorbed copper passed through the column, the appearance of the resin changed towards a slightly deeper color. Therefore the collection of the desorbed copper was only began when the acid front is ~0.5 cm from the end of the column. From this moment on, 3.5 ml solution which contained all the

TABLE IVa

In Vitro ^{64}Cu Labelled Human Blood Serum on Chelex 100 Resin Column.

Sample Size: 2 ml.

Resin mesh size	Column size d x l cm	Flow rate ml /hr	% of ^{64}Cu NH_4OAc fraction	HCl fraction
100-200	0.6 x 10	20	56	44
	0.6 x 15	15	42	58
200-400	0.6 x 10	6.0	40	60
	0.6 x 16	5.7	23	77
	0.6 x 20	5.0	21	79
	0.6 x 25	4.3	19	81
	0.6 x 30	4.1	13	87
	0.6 x 26	2.0	2.0	98

TABLE IVb

<u>In Vitro</u> ^{64}Cu Labelled Human Blood Serum on Chelex 100 Resin Column.

Resin mesh size: 200 - 400

Length of columns: 26 - 28 cm.

Flow rate: 2.0 - 2.4 ml/hr.

Weight of Serum Samples (g)	% of ^{64}Cu	
	NH_4OAc fraction	HCl fraction
1.44	1.16	98.84
2.01	2.01	97.99
2.46	1.28	98.72
3.00	1.70	98.30
3.54	1.29	98.71
4.50	2.10	97.90
5.30	5.54	94.46

copper orginally adsorbed on the Chelex 100 resin was collected. This was established through the elution curve shown in Figure II.

These experiments show that the non-ceruloplasmin bound copper can be isolated from the blood serum. They also show the comparatively strong binding of copper by albumin; as the same amount of copper (<<1 μg) can be removed from an albumin free aqueous solution by a few mg of the resin.

3.2. Behaviour of Histidine-Copper Complex on Chelex 100 Resin Column..

Neumann and Sass-Kortsak[39,40] showed that there is a third small fraction of copper in human blood serum which is bound to amino acids, in addition to that bound to ceruloplasmin and albumin. Histidine is the one primarily involved in this type of copper amino acids complex. Copper may be bound to two histidine molecules or one histidine and another amino acid such as threonine, glutamine or asparagine. In order to study the behaviour of this type of copper amino acid complex on the Chelex 100 resin column to see what effect if any it may have on the separation scheme, the following experiments were done..

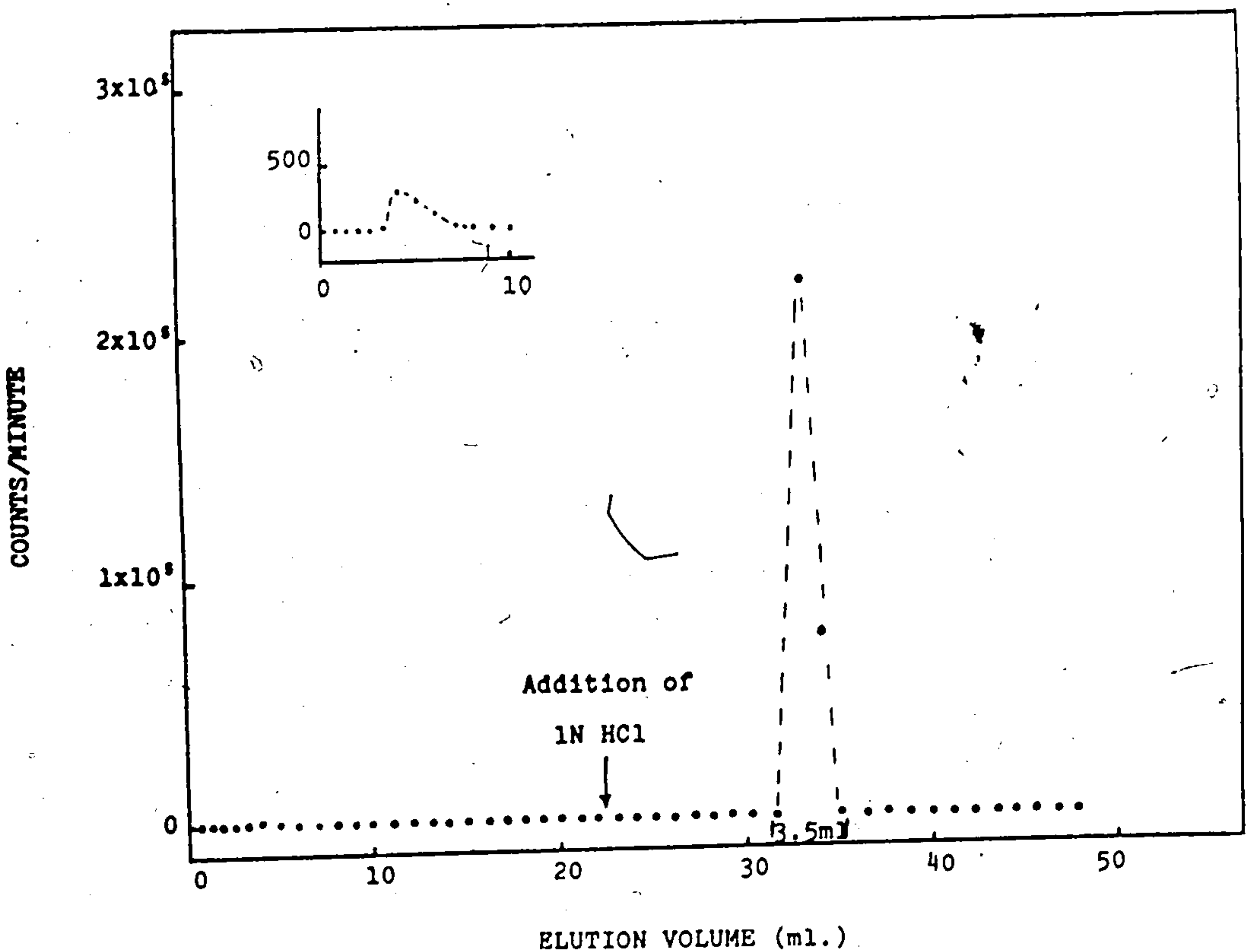
^{64}Cu labelled histidine-copper complex was prepared by mixing histidine and copper solution. Three sample solutions were prepared in this way with different molar ratio of histidine to copper. Solution A contained 21 mg histidine/l and 70 μg copper/l. These are the amounts of histidine and non-ceruloplasmin bound copper

FIGURE II

Human Blood Serum Spiked with ^{64}Cu on Chelex 100 Resin Column (0.6 x 27 cm flow rate 2 ml/hr).

Sample size: 2.5 ml.

The Small Figure at the Left Top Corner is an Enlargement of the First 10 ml of Eluant.


3x10^5
2x10^5
1x10^5
0
COUNTS/MINUTE
500
0
0
10
Addition of
1N HCl
3.5ml
0
10
20
30
40
50
ELUTION VOLUME (ml.)

present in human blood serum.[18] The molar ratio of histidine to copper is 1300:11. In solutions B and C, the molar ratios were 2:1 and 1000:1, respectively, but they contained a higher concentration of both histidine and copper (0.90 g Histidine/l). This was found to be necessary to identify the histidine by the color developed with ninhydrin. Thin layer chromatography and Chelex 100 resin column chromatorgraphy were carried out simultaneously with the solutions.

In the thin layer chromatography experiment, silica gel thin layer sheet (2 x 20 cm) was used. Approximately 0.1 ml solution was applied to the sheet about 4 cm above the bottom edge of the sheet. 60% CH_3COCH_3/H_2O was used as the eluating solvent in ascending chromatography. The histidine was identified by spraying the sheet with ninhydrin solution. A pink-purple color spot was observed with the sheets for solutions B and C, but not for solution A due to the small amount of histidine present. The sheet was then cut into several pieces and the ^{64}Cu activity was measured with the 3 x 3 inch NaI(Tl) crystal. Duplicate experiments were carried out. Figure III shows the three sets of the results. It can be seen in Figure IIIb and Figure IIIc that over 50% of the ^{64}Cu activity is found in the pink-purple spot. This indicates the formation of histidine-copper complex.

When the solutions were put on the Chelex 100 resin columns, it is found that, for all the three solutions,

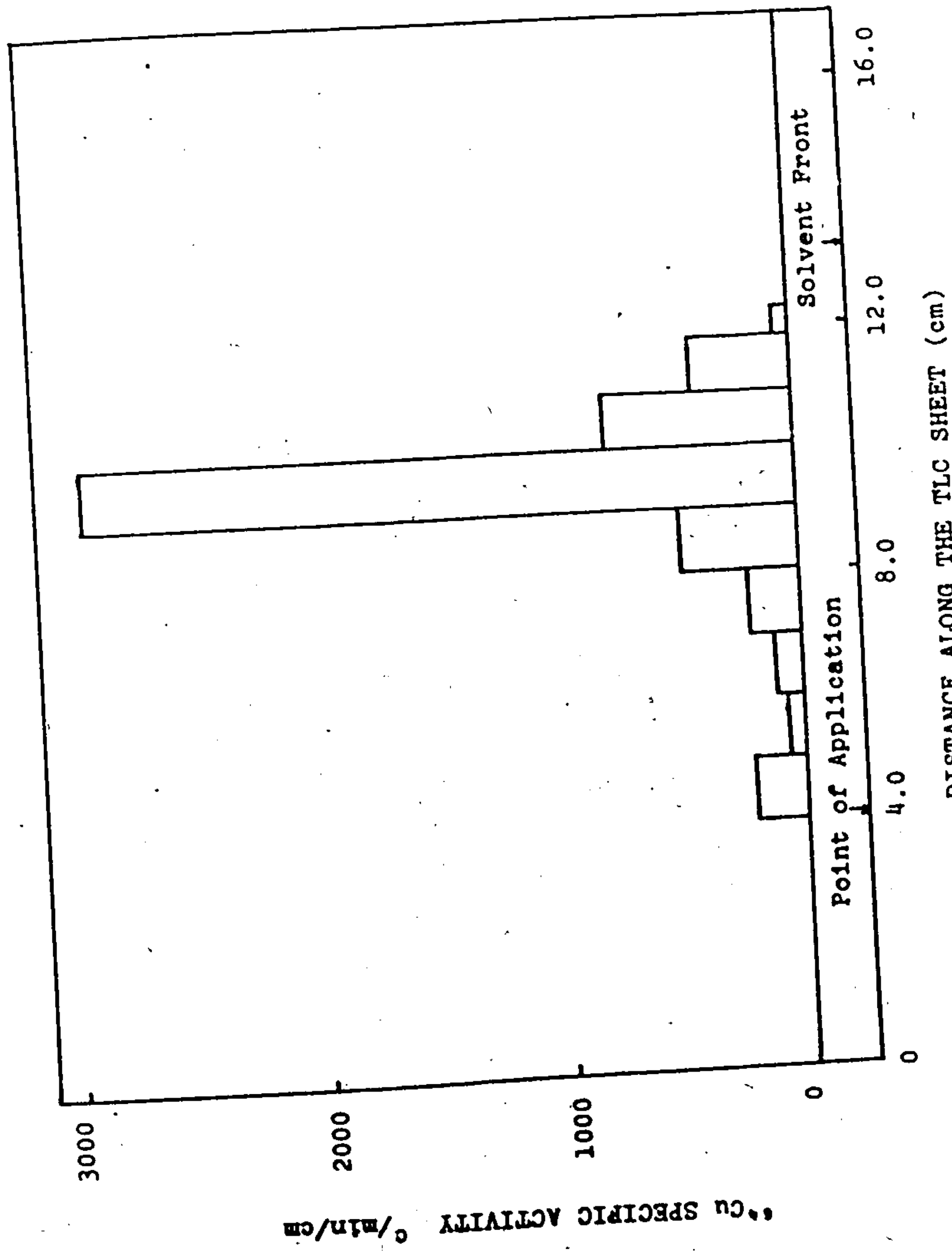
FIGURE IIIa

Thin Layer Chromatography of Histidine-Copper Complex.

Solution A

Histidine/Copper = $1.3 \times 10^{-4}/1.1 \times 10^{-6}$

(molar ratio)



Solvent Front
Point of Application
DISTANCE ALONG THE TLC SHEET (cm)
0
4.0
8.0
12.0
16.0
64Cu SPECIFIC ACTIVITY c/min/cm
0
1000
2000
3000

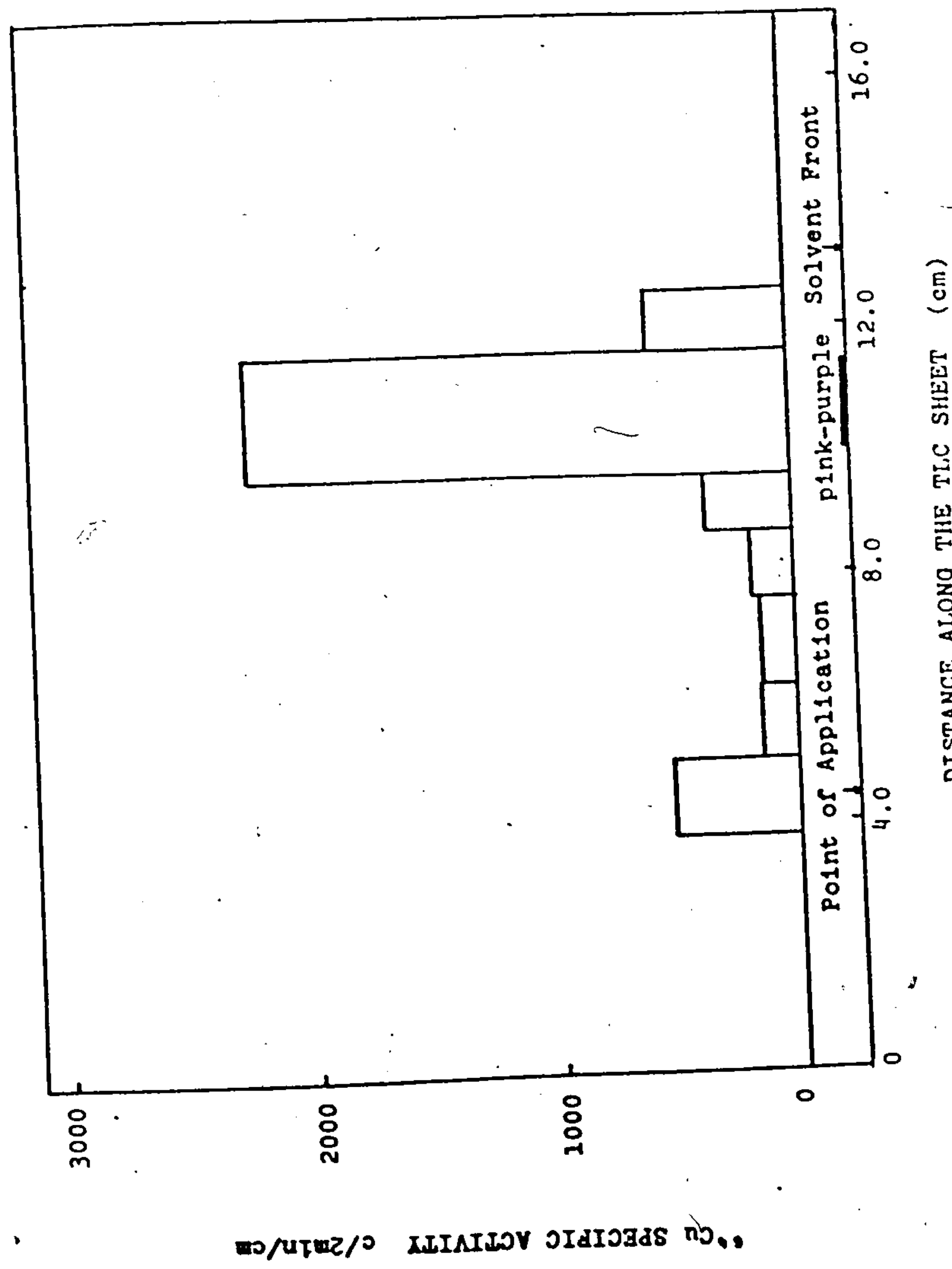
FIGURE IIIb

Thin Layer Chromatography of Histidine-Copper Complex.

Solution B

Histidine/Copper = $5 \times 10^{-3}/2.4 \times 10^{-3}$

(molar ratio)



Point of Application
pink-purple
Solvent Front
0
4.0
8.0
12.0
16.0
DISTANCE ALONG THE TLC SHEET (cm)
0
1000
2000
3000
^{64}Cu SPECIFIC ACTIVITY c/2min/cm

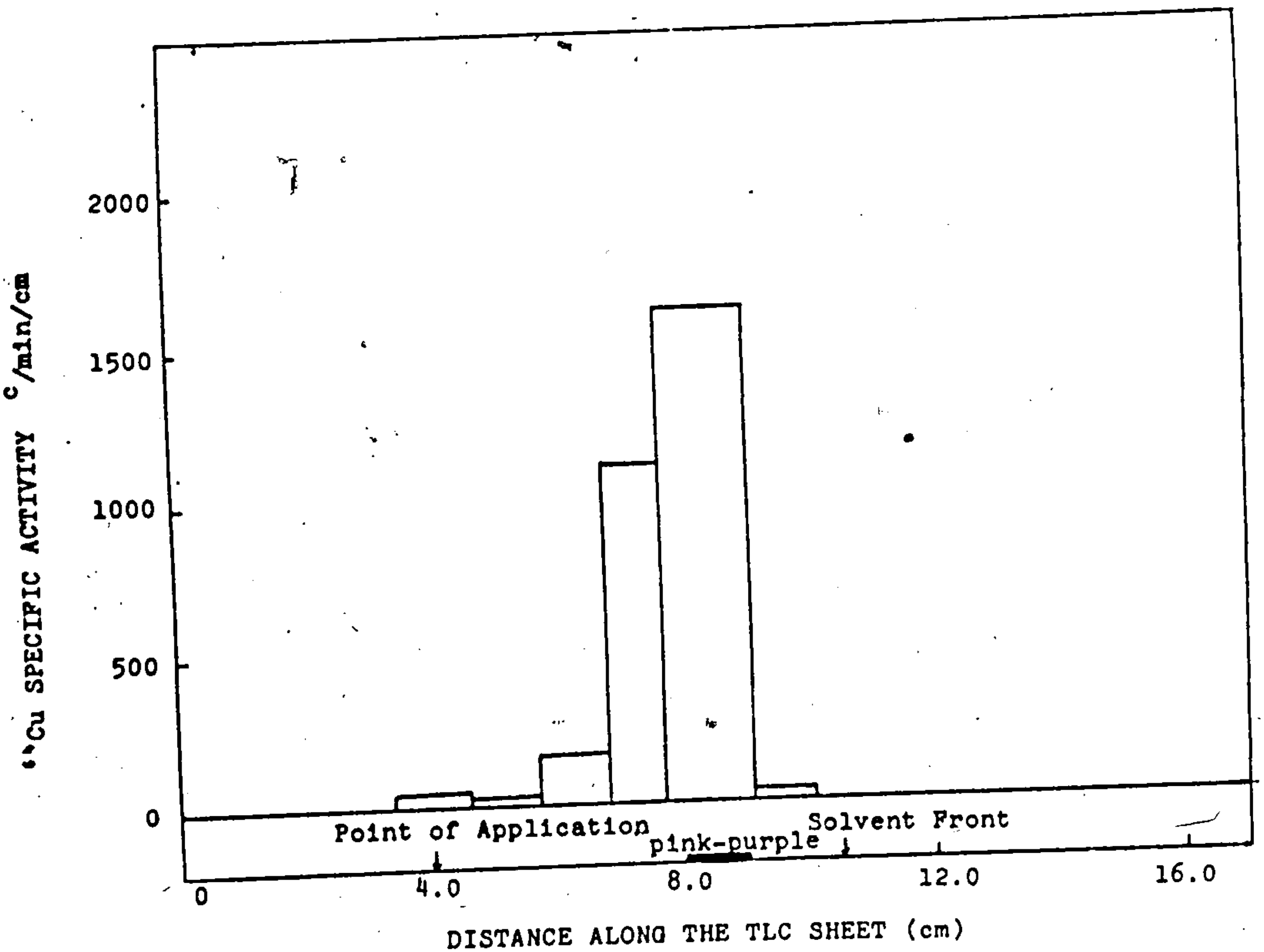
FIGURE IIIc

Thin Layer Chromatography of Histidine-Copper Complex.

Solution C

Histidine/Copper = $5 \times 10^{-3} / 5 \times 10^{-6}$

(molar ratio)


2000
1500
1000
500
0
^{64}Cu SPECIFIC ACTIVITY ^{c}/min/cm
Point of Application
pink-purple
Solvent Front
0
4.0
8.0
12.0
16.0
DISTANCE ALONG THE TLC SHEET (cm)

>99.5% of the ^{64}Cu was retained by the resin and later collected in the hydrochloric acid fraction. This indicates that the copper in the histidine-copper complex can be adsorbed on the resin and can be separated from the copper bound to ceruloplasmin. In other words, it behaves in the same way as the copper bound to albumin.

3.3. <u>In Vivo Addition of Copper-64 to Rat Serum</u>.

It has been shown that following the injection or intravenous administration of ^{64}Cu to human, there is a rapid increase in plasma radioactivity followed by a rapid fall; then there is another marked and sustained secondary rise in total plasma activity.[56,57,167-171]

During the early stages, the ^{64}Cu in blood serum is bound almost exclusively to albumin. In the secondary rise of activity, however, most of the ^{64}Cu is bound to ceruloplasmin discharged from the liver.

The same phenomenon was also observed with a rat.[173] This makes it possible to study the behaviour of ceruloplasmin bound copper on the Chelex 100 resin column under the conditions established in the *in vitro* experiment. About 1 m Ci carrier-free ^{64}Cu was injected intravenously into a rat. Blood was obtained by cardiac puncture twenty-four hours later. It is known that almost all of the radioactive ^{64}Cu found in the serum is incorporated into the ceruloplasmin at this stage.[172] One fraction of the serum was chromatographed on the Bio-Gel P-150 column, while the remaining was divided and put on three Chelex 100 resin columns.

Protein fractionation with the Bio-Gel P-150 is shown in Figure IV. It can be seen that the ^{64}Cu activity appeared in a single peak with a tailing to the albumin region. This peak represents the ceruloplasmin.[173,174] Upon resolving the peak into two Gaussian-shaped curves, with the DuPont 310 Curve Resolver, it is found that approximately 96% of the total ^{64}Cu activity is contributed from the ceruloplasmin bound copper.

For the separation of the non-ceruloplasmin bound copper from ceruloplasmin bound copper on the Chelex 100 resin columns, the result is shown in Table V. 95% (average of three results) of the total ^{64}Cu activity is found in the ceruloplasmin fraction. This agrees quite well with the result from gel chromatography.

Further justification of the method of separation is obtained by repeating the experiment with some modification. *In vivo* ^{64}Cu labelled rat blood serum was obtained as before. The eluate from the Chelex 100 resin column was collected by a fraction collector and the ^{64}Cu activity in each fraction was measured. Figures V and VI show the chromatograms for the two samples. It can be seen that the ^{64}Cu bound to ceruloplasmin is not adsorbed on the resin and passes through the column directly.

The chromatogram for the protein fractionation of the serum with the Bio-Gel P-150 column is shown in Figure VII.

FIGURE IV

Bio-Gel P-150 Chromatography of _In Vivo_ ^{64}Cu Labelled Rat Blood Serum.

Sample Size: 0.75 g.

Column Size: 1.6 x 35 cm.

Protein Distribution Curve - Solid Line

^{64}Cu Activity Curve - Dot Line

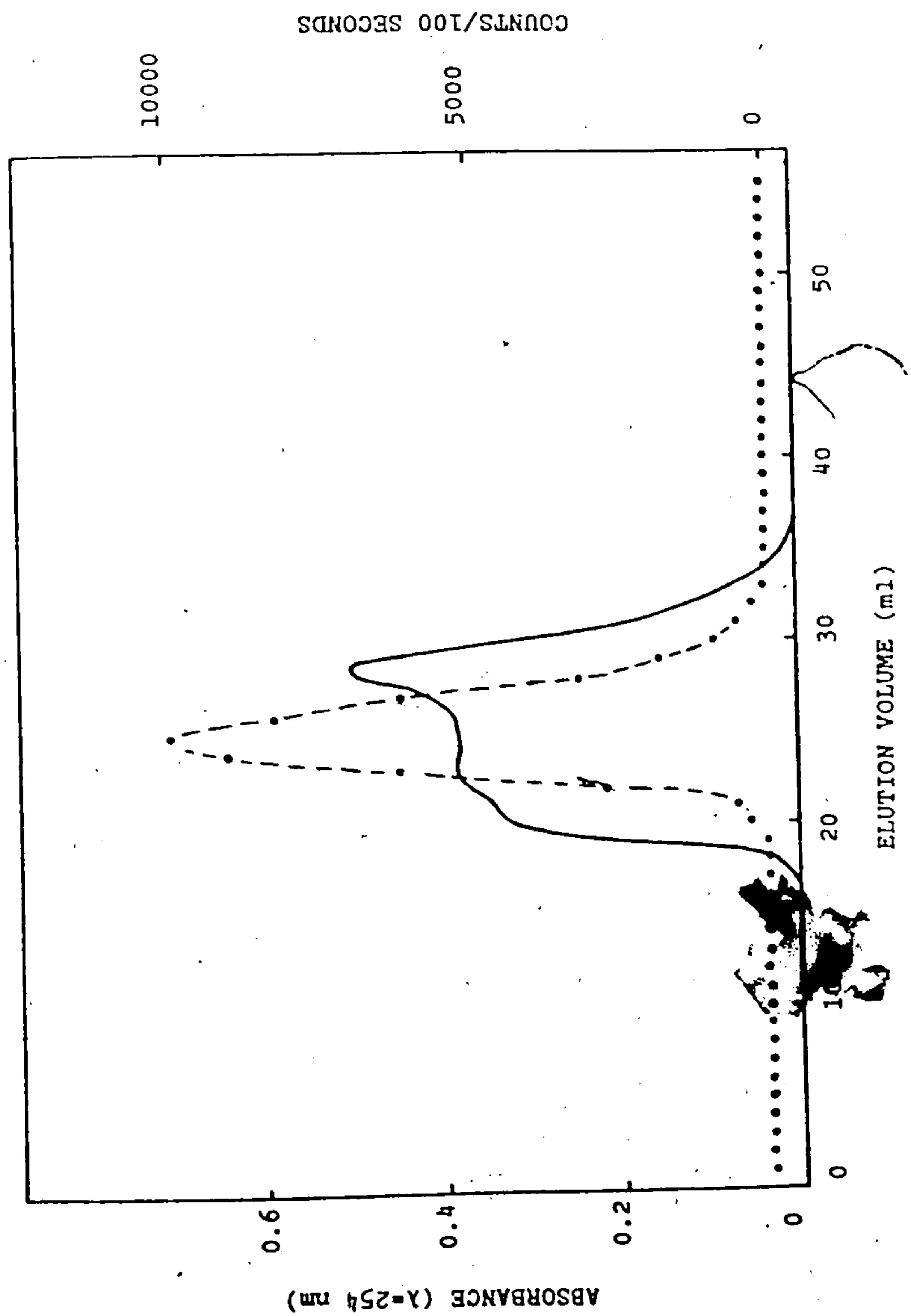

COUNTS/100 SECONDS
10000
5000
0
ELUTION VOLUME (ml)
0
10
20
30
40
50
ABSORBANCE (λ=254 nm)
0.6
0.4
0.2
0

TABLE V

In Vivo ^{64}Cu Labelled Rat Blood Serum on Chelex 100 Resin Columns.

	% of ^{64}Cu	
Column No.	HCl Fraction (Non-ceruloplasmin Bound Copper)	NH_4OAc Fraction (Ceruloplasmin Bound Copper)
1	5.1	94.9
2	4.9	95.1
3	5.0	95.0
Average	5.0	95.0

FIGURE V

Chelex 100 Resin Chromatography of *In Vivo* ^{64}Cu Labelled Rat Blood Serum (Sample No. 1).

Sample Size: 0.6 g.

Column Size: 0.6 x 27 cm.

Flow Rate: 2.2 ml/hr.

(The area under the curve as shown on the graph corresponds to the amount of ^{64}Cu activity.)

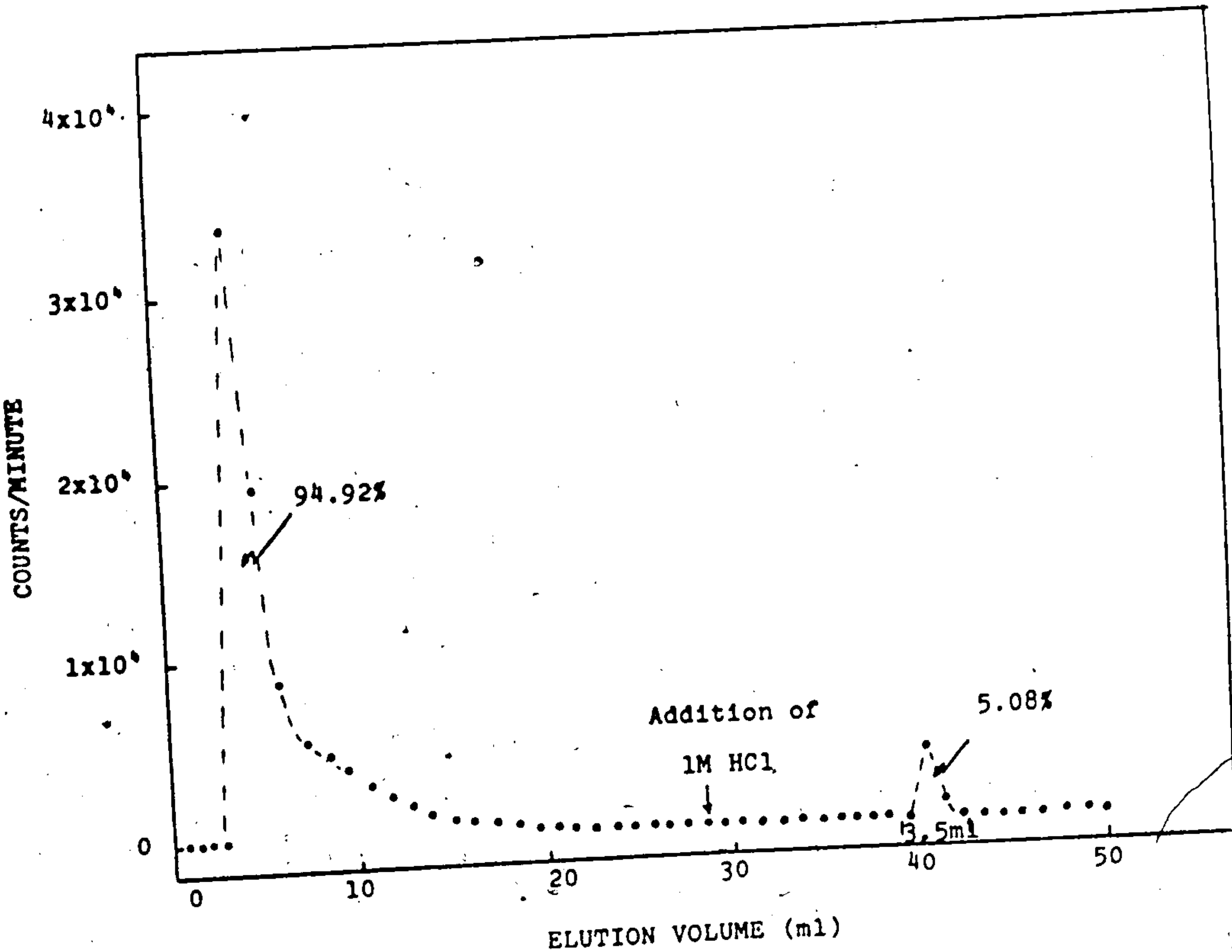

4x10⁴
3x10⁴
2x10⁴
1x10⁴
0
COUNTS/MINUTE
94.92%
Addition of
1M HCl
5.08%
3.5ml
0
10
20
30
40
50
ELUTION VOLUME (ml)

FIGURE VI

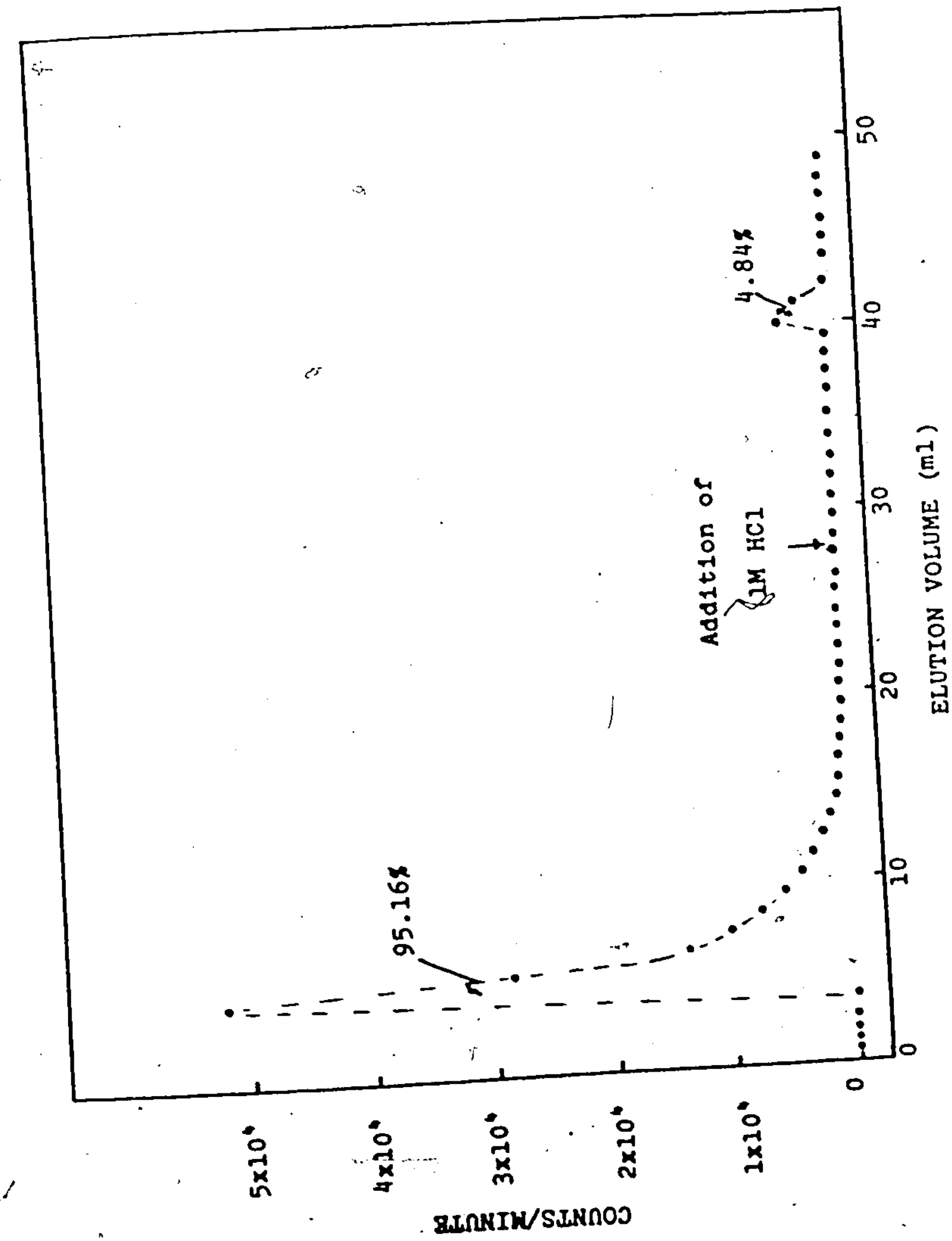
Chelex 100 Resin Chromatography of In Vivo ^{64}Cu Labelled Rat Blood Serum (Sample No. 2).

Sample Size: 1.3 g.

Column Size: 0.6 x 28 cm.

Flow Rate: 2.0 ml/hr.

(The area under the curve as shown on the graph corresponds to the amount of ^{64}Cu activity.)



COUNTS/MINUTE
5x10⁴
4x10⁴
3x10⁴
2x10⁴
1x10⁴
0
ELUTION VOLUME (ml)
0
10
20
30
40
50
95.16%
4.84%
Addition of 1M HCl

FIGURE VII

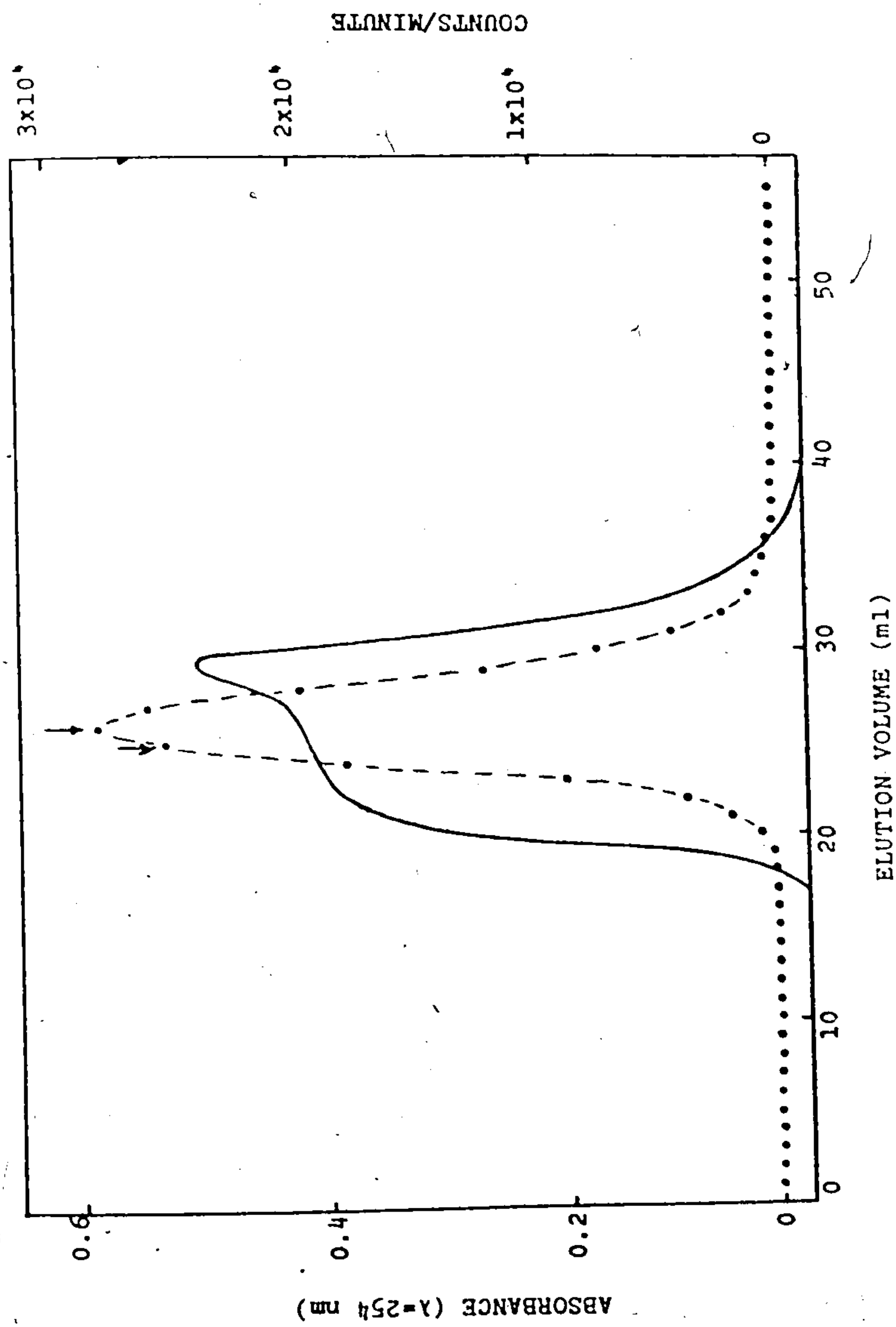
Bio-Gel P-150 Chromatography of <u>In</u> <u>Vivo</u> ^{64}Cu Labelled Rat Blood Serum.

Sample Size: 1.07 g.

Column Size: 1.6 x 35 cm.

Protein Distribution Curve - Solid Line

^{64}Cu Activity Curve - Dot Line


COUNTS/MINUTE
3x10
2x10
1x10
0
0.6
0.4
0.2
0
ABSORBANCE (λ=254 nm)
0
10
20
30
40
50
ELUTION VOLUME (ml)

Two of the fractions (marked with an arrow) containing ceruloplasmin, but no albumin or amino acids, were combined and then passed over a third Chelex 100 resin column. No ^{64}Cu was retained by this column.

The in vitro and in vivo experiments together show that the ceruloplasmin bound copper passes freely through the Chelex 100 resin column whereas the non-ceruloplasmin bound copper is quantitatively (>98%) retained. Thus it provides a method to separate the two forms of bound copper from each other and these can be determined later.

3.4. Methods to Prevent Contamination.

In the determination of the non-ceruloplasmin bound copper, the blank has to be kept as low as possible. In these studies, about 3 g of human blood serum are usually used. This corresponds to 15 - 30 x 10^{-8} g of non-ceruloplasmin bound copper, which accounts for 5 - 10% of the total copper in serum. Thus, the blank for the separating procedures has to be kept below 1.5 x 10^{-8} g copper to make it suitable for the experiment.

In order to attain this, great precautions were taken to avoid contamination. First of all, a plastic syringe was used instead of a vacuum glass tube, to draw blood from volunteers. The possibility that the plastic syringe and hypodermic needle may introduce contamination was also tested. Different amounts of deionised distilled water were drawn into several syringes and later delivered into polyethylene bottles for freeze-drying and analysis for copper by neutron activation analysis. The results obtained showed no detectable amount of copper (<0.1 ppb). Moreover, five fractions of a copper standard solution (105 ppb), instead of deionised water, were also drawn into syringes. Complete recovery of the copper after delivering the solutions into the polyethylene bottles was also obtained. This proves the plastic syringes and hypodermic needles were good for the purposes of this experiment. A recent study by Versieck and Speecke[175] also justifies this result.

The blood sample was immediately transferred into a quartz centrifuge tube for coagulation. The tube had been soaked in aqua regia for twenty-four hours and thoroughly rinsed with deionised distilled water before use.

The dilute hydrochloric acid and ammonium acetate solution used in the separation were prepared from B.D.H. Aristar grade hydrochloric acid, ammonia and acetic acid. The resulting solutions contained less than 1 ppb. copper as determined by neutron activation analysis.

The commercially available Chelex 100 resin contains some copper. This prevents it from direct use without purification. The method for the purification of the resin and further precautions in the separation of the two different forms of bound copper were mentioned in section 2.1. and 2.5., respectively.

The experiments were carried out inside a plexiglass box in a clean laboratory. Under these conditions, the procedure blank was 1 - 1.5 x 10^{-8} g copper in the 3.5 ml HCl eluant, and this was very reproducible throughout the studies.

3.5. Determination of Copper in Blood Serum by Neutron Activation Analysis.

In most of the work present here, both total copper and non-ceruloplasmin bound copper in blood serum were determined by neutron activation analysis. The sensitivity of the method suggested by Kaiser and Meinke[68] is too poor. The repeated precipitations method suggested by Bowen[59] is tedious. Fritze *et al*.[177] suggested the use of ion-exchange chromatography for the isolation of the ^{64}Cu activity. The method suggested in this study (section 2.6.1. and 2.7.) is a simplification of the one used by Fritze *et al*. It is quite simple and straightforward. The pH of the solution added to the resin column is not critical provided that it is greater than 3. Chemical yield determination is not necessary since the recovery is quantitative. Moreover, the method may be used to determine copper in many different biological samples and materials other than blood serum.

In this study, a pooled serum sample from five volunteers (three males and two females) was analyzed repeatedly for the total copper and the non-ceruloplasmin bound copper. The results are given in Table VI. The sample sizes were determined by weight instead of volume. The weights ranged

TABLE VI

Determination of Non-ceruloplasmin Bound Copper and Total Copper in a Pooled Human Blood Serum by Neutron Activation Analysis.

Non-ceruloplasmin bound copper µg/100 g	Total copper µg/100 g
6.4	105
6.0	106
6.1	108
6.2	104
6.2	105
5.7	102
6.1	107
5.9	101
6.2	103
5.8	103
6.0	102
6.1 ± 0.2	104 ± 2

from 1.8 to 3.2 g for the determination of non-ceruloplasmin bound copper, and 0.7 to 1 g for the determination of total copper. Reagent blanks were run in parallel and amounted to 5 - 10% of the copper in the sample depending on the sample sizes, for the non-ceruloplasmin bound copper determination. As can be seen, the reproducibility is good. From the data given, it can be calculated that the ceruloplasmin bound copper is equal to 98 ± 2 μg copper/100 g serum, which accounts for 94.2% of the total copper in blood serum. Since a small number (the non-ceruloplasmin bound copper) is subtracted from a large one (the total serum copper), the result will be accurate, namely, the error is the same as in the determination of the total copper.

3.6. Determination of Copper in Blood Serum by Atomic Absorption Spectroscopy.

In the determination of total copper in blood serum, several methods are available to prepare the solution to be sprayed into the flame of the atomic absorption instrument. Sprague and Slavin[103] suggested a simple 1:1 dilution of the serum with water. The calibration curve is obtained from aqueous standard solutions. This method of analysis is found to give low and irreproducible results.[105] The discrepancy is partially due to the higher viscosity of the diluted serum, which impairs the aspiration of the sample into the burner. The different status of copper: protein bound copper in the serum sample, but aqueous ionic copper in the standards, also contributes to the low results. Moreover, the serum sample may also cause clogging of the aspirating tube and burner unless a "high solid" burner head is used.[106]

Olson and Hamlin[107] suggested the proteins in the serum to be precipitated by 20% trichloroacetic acid. The clear supernatant was then used for analysis for copper. In these studies, pig plasma was used to study this method. To 0.5 ml pig plasma an equal volume of 20% trichloroacetic acid was added. In order to get a higher atomic absorbance signal, the copper in the supernatant was extracted into methyl isobutyl ketone with ammonium pyrrolidine dithiocarbamate as the chelating agent. (This extraction step was found to be quantitative as described later in this section.) The absorbance of the organic layer in the flame was then measured. Four determinations each were done on two different plasma samples. For the first sample, the following absorbance readings were obtained: 0.061, 0.054, 0.067 and 0.068 (0.063 $\pm$ 0.006). For the second sample, the absorbance readings were: 0.087, 0.078, 0.073 and 0.081 (0.080 $\pm$ 0.006). The large ranges of the two analyses, 0.014 on an average value of 0.063 and 0.014 on an average value of 0.080 respectively, show that the method is not very precise. The reason for the irreproducibility may be due to the incomplete liberation of copper from the proteins when they are precipitated by trichloroacetic acid. Thus different amounts of copper are lost from the samples during the process.

The liberation of copper from plasma or whole blood with hydrochloric acid was first demonstrated by Holmberg.[178] Gubler et al.[127] suggested the use of hydrochloric acid, together with trichloroacetic acid, to liberate the copper and precipitate the serum proteins before the copper was determined spectrophotometrically. This method of liberation of copper from the serum proteins was found to give quantitative and reproducible results in these studies. The result agrees with that obtained by wet digestion of the serum with sulfuric and nitric acids.

This method is simple, fast and accurate. It also provides a slightly acidic solution of small volume. The copper in it can be extracted into an organic solvent for the measurement of absorbance in both atomic absorption and spectrophotometric analyses.

Several advantages result when solvent extraction is used for atomic absorption analysis:

1. The sensitivity for all metals is enhanced 300 - 500% when the metal is determined in an organic solvent. This is due to the formation of a readily volatile compound, lower viscosity of the organic solvent, and a higher atomization efficiency,[179] (the ratio of the amount of solution passing to the burner to the total amount of solute atomized).

2. By adjusting the volume ratio between the aqueous and organic phase, the metal can be concentrated in the organic phase.

3. The salt content of a solution used for extraction can be far greater than that of one which can be handled by any burner. The interfering materials can be left behind in the aqueous phase. For instance, the interferences from sodium, potassium and calcium in the copper determination can be eliminated by extracting copper into methyl isobutyl ketone.

Allan[96] found that the sensitivity of the copper determination could be increased several fold by complexing the copper with ammonium pyrrolidine dithiocarbamate (APDC) prior to extraction into methyl isobutyl ketone (MIBK) or ethyl acetate. This complexing reagent, the preparation of which has been described by Malissa and Schoffmann,[100] has been preferred for two reasons. Firstly, the copper complex is readily soluble in the types of organic solvents which could be successfully aspirated into the flame. Secondly, the complex can be formed and extracted easily. The only disadvantage of the reagent is that a fresh solution has to be prepared before use. Figure VIII shows the increase of sensitivity by using this extraction method.

In these studies, by using ^{64}Cu as a tracer, it is found that all the copper (1 ng to 10 μg) can be extracted quantitatively into 1.50 ml methyl isobutyl ketone with 100 μl of 1% aqueous ammonium pyrrolidine dithiocarbamate solution. The extraction is essentially pH independent. Total recovery can be obtained from solutions of pH ~8 to 3N HCl. When the

FIGURE VIII

Typical Calibration Curve for Copper Standard Solutions by Atomic Absorption Spectroscopy.

λ = 3247.5 Å

Lamp Current: 12 mamp.

Air-Acetylene Flame with a 10 cm Light Path Length

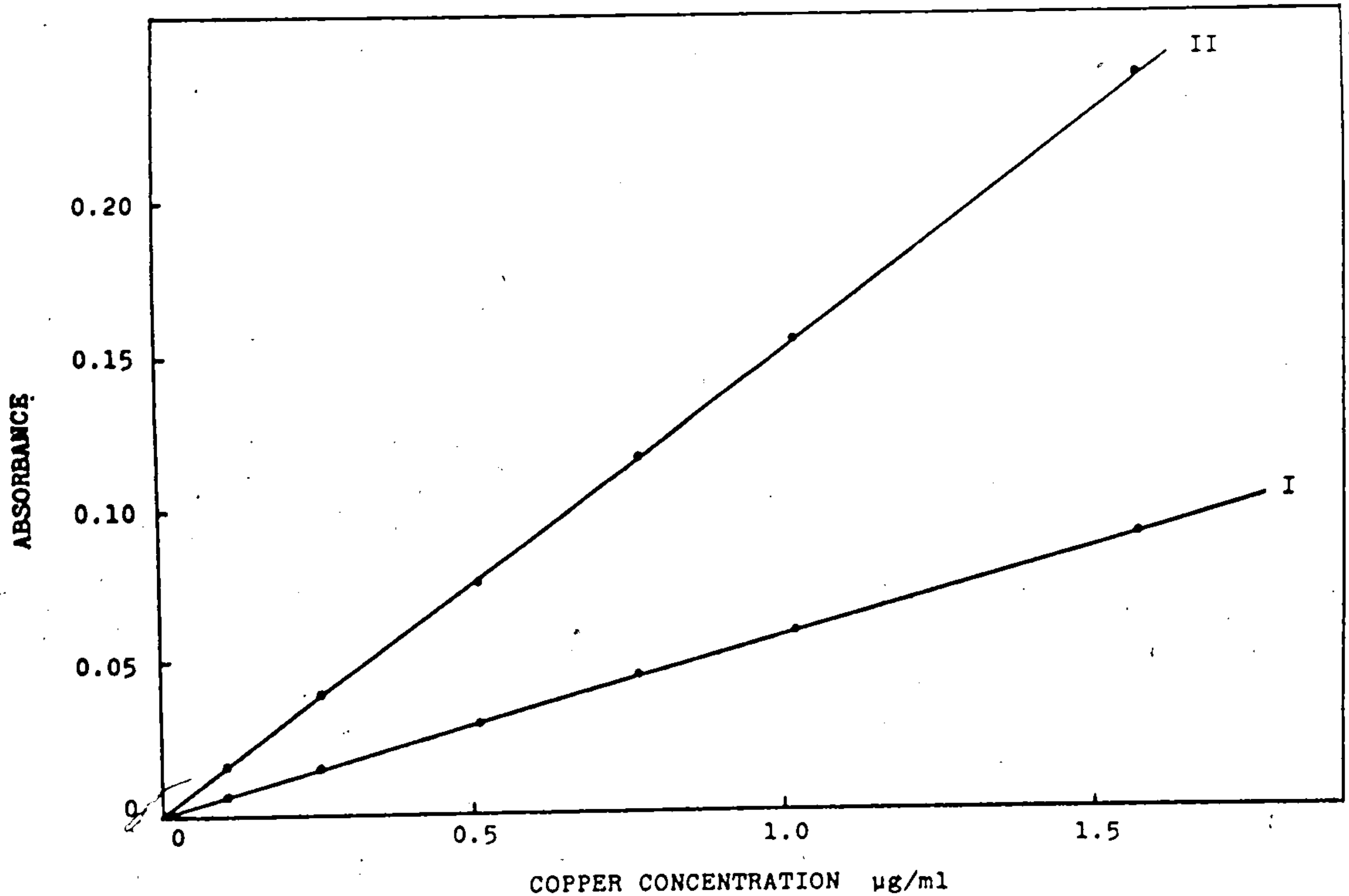
Premix Burner.

Line I: Aqueous Copper Standard Solution.

Line II: Copper Ammonium Pyrrolidine Dithiocarbamate in

Methyl Isobutyl Ketone.

(This plot shows that both the copper solutions obey Beer's Law in the range of concentration shown in the graph.)


II
I
ABSORBANCE
0.20
0.15
0.10
0.05
0
0
0.5
1.0
1.5
COPPER CONCENTRATION μg/ml

organic layer is sprayed into the flame of the atomic absorption unit, no interference from other ions is found by using an ac system and a premix air-acetylene flame.

In the determination of total copper in blood serum, the serum proteins are precipitated by hydrochloric acid and trichloroacetic acid. An experiment with ^{64}Cu as tracer demonstrated that trichloroacetic acid does not affect the extraction efficiency of copper into methyl isobutyl ketone, but the absorbance reading in the atomic absorption unit is reduced by about 20% (see Figure IX). Thus the same amount of trichloroacetic acid must be added to the aqueous standard solutions prior to extraction to eliminate a systematic error.

In order to study the reproducibility of this method of analysis for copper, a pooled pig plasma sample was analysed repeatedly for total copper by atomic absorption spectroscopy. The procedure for this method has been described in section 2.7. Table VII gives the results of this study. An average of 0.845 μg/500 μl is obtained with a standard deviation of 0.013 μg/500 μl. The reproducibility is satisfactory.

Another pooled pig plasma sample was analysed repeatedly by both neutron activation analysis and atomic absorption spectroscopy. The results are shown in Table VIII. Neutron activation analysis gives a result of 1.961 ± 0.025 ppm., whereas atomic absorption spectroscopy gives 1.912 ± 0.051 ppm. By comparing the two results with the t test,[189,190] t is found to be 0.563. This value of t is less than the critical

FIGURE IX

Effect of Trichloroacetic Acid on the Determination of Copper by Atomic Absorption Spectroscopy.

$\lambda = 3247.5$ Å

Lamp Current: 12 mamp.

Air-Acetylene Flame with a 10 cm Light Path Length Premix Burner.

Line I: 1.00 ml Cu Standard Solution + 1.00 ml 3N HCl + 1.00 ml 20% Trichloroacetic Acid + 100 µl 1% Aqueous Ammonium Pyrrolidine Dithiocarbamate Extracted into 1.50 ml Methyl Isobutyl Ketone.

Line II: 1.00 ml Cu Standard Solution + 1.00 ml 3N HCl + 1.00 ml H_2O + 100 µl 1% Aqueous Ammonium Pyrrolidine Dithiocarbamate Extracted into 1.50 ml Methyl Isobutyl Ketone.

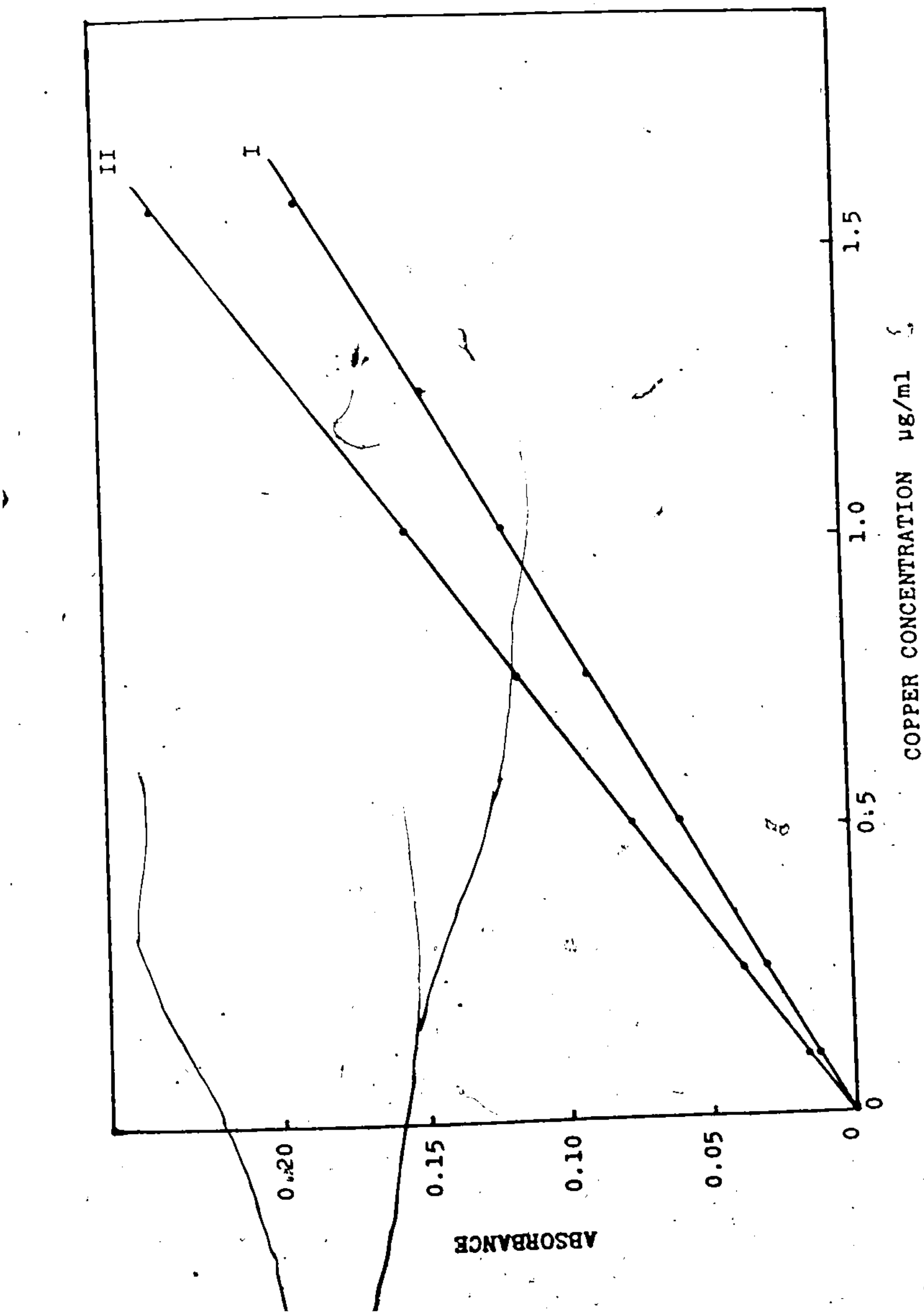

ABSORBANCE
0
0.05
0.10
0.15
0.20
0
0.5
1.0
1.5
COPPER CONCENTRATION µg/ml
I
II

TABLE VII

Determination of Total Copper in a Pooled Pig Plasma Sample by Atomic Absorption Spectroscopy.

Sample Size: 500 µl.

No.	Copper Concentration µg/500 µl
1	0.866
2	0.855
3	0.855
4	0.847
5	0.836
6	0.836
7	0.855
8	0.836
9	0.826
10	0.836
	0.845 ± 0.013

TABLE VIII

Determination of Total Copper in a Pooled Pig Plasma Sample by Neutron Activation Analysis (NAA) and Atomic Absorption Spectroscopy (AAS).

Copper Concentration μg/ml	
NAA	AAS
1.992	1.944
1.961	1.892
1.931	1.866
1.960	2.002
	1.920
	1.892
	1.840
	1.944
1.961 ± 0.025	1.912 ± 0.051

value of t at 5% probability level (2.228). It then may be concluded that the two methods of analyses agree well with each other.

As for the determination of non-ceruloplasmin bound copper, it is quite simple and straightforward after separation of the two different forms of bound copper on the Chelex 100 resin column. The procedure for the analysis has been described in section 2.6. Two pooled pig plasma samples were used to analyze for the two different forms of bound copper by neutron activation analysis, atomic absorption spectroscopy and also by a spectrophotometric method described in the next section. The results are shown in Table X and Table XI, and these will be discussed in section 3.8.

3.7. Determination of Copper in Blood Serum by a Spectrophotometric Method.

Spectrophotometric analysis for copper has the advantage of not requiring expensive apparatus. The sensitivity range of present-day spectrophotometric methods is adequate for most purposes; their specificity and accuracy are also acceptable.

Table III shows the most important and common color reagents for the determination of copper. Their relative sensitivities can be compared on the basis of their molar absorptivities. Eden and Green[123] chose diethyldithiocarbamate as the color reagent. Wet ashing with a combination of sulfuric, perchloric and nitric acid was employed by them for the destruction of organic matter, followed by complexation of the iron with citrate or pyrophosphate in strongly alkaline medium. The copper complex was then extracted into amyl alcohol for absorbance measurement. A problem with this reagent is that the extraction of the copper diethyldithiocarbamate complex gives irregular and low recovery of added copper at low pH values.[177] This may be explained as follows: a low pH inhibits complex formation between metal and ligands in the aqueous phase; preferential extraction of reagent into the solvent layer takes place before appreciable chelation can occur; and inactivation or decomposition of the reagent occurs prior to chelation. Thus the copper extraction is usually carried out in strongly alkaline medium. Under this condition, iron interferes strongly by forming the highly brown color ferric diethyldithiocarbamate due to the large amount of iron present in blood (1.14 mg/l plasma, 1110 mg/l red cells).[176] This can be prevented by complexing the iron with citrate or pyrophosphate. The many reagents used in the procedure greatly enhance the possibilities of contamination. Extreme care must be taken to keep the blank low.

Gubler et al.[127] suggested the use of dilute hydrochloric acid to liberate copper from serum proteins prior to precipitation of the proteins by trichloroacetic acid, instead of wet digestion of the serum. Diethyldithiocarbamate was again used as the color reagent together with citrate and pyrophosphate in alkaline medium as in Eden and Green's method,[123] but the absorbance was measured directly in the aqueous solution. This method simplifies the one suggested by Eden and Green, but does not help to improve the contamination problem. Furthermore, the sensitivity of this method is much less due to the lower molar absorptivity of the copper diethyldithiocarbamate complex in aqueous solution than in an organic solvent and the complex is in colloidal suspension in water, which makes absorbance reading uncertain.[124,128]

Dithizone is a very versatile complexing reagent. It forms colored compounds with a great number of metals besides copper. The extraction step requires a fairly long period of shaking. The metal dithizonates and dithizone itself decompose rapidly if exposed to strong light or subjected to relatively high temperature. Strong oxidizing agents also destroy dithizone and they must therefore be removed by the use of hydroxylamine, hydrazine or ascorbic acid before an extraction step. Extraction of the copper dithizonate may provide a means of separation, but the method based on use of dithizone alone is unsuitable because of its lack of specificity.

2, 2'-Biquinoline (cuproine) and 2, 9-dimethyl-1, 10-phenanthroline (neocuproine) are specific for Cu(I). Hydroxylamine hydrochloride is a suitable reducing agent and tartrate is used to prevent precipitation of various metals. The former cuprous complex can be extracted into amyl alcohol (iso- or n-) within pH range of 4.5 - 7.5, while the latter can be extracted into $CHCl_3$-EtOH at pH 2 - 9. The disadvantage of these reagents is their low sensitivities. They are not suitable for determination of copper below 1 μg.

2, 9-Dimethyl-4, 7-diphenyl-1, 10-phenanthroline (bathocuproine) is also highly specific for Cu(I). The copper chelate is readily extractable into hexanol with a single extraction and it is stable for four days. Other metals do not form colored complexes under the reaction conditions specified. It also has a higher molar absorptivity than cuproine and neocuproine. Consequently, the reagent is more suitable for microanalysis for copper. The drawback of this reagent is that hydroxylamine hydrochloride must be added to reduce the copper to the cuprous state after the digestion step. Ammonium hydroxide is also required to neutralize the solution. Moreover, this reagent is quite expensive in comparison with other spectrophotometric reagents (1 g/$35).

Biscyclohexanoneoxalyldihydrazone (cuprizone) was suggested by Peterson and Bollier.[128] It gives a blue coloration with Cu(II) at pH 7 - 9 in a citrate medium (to prevent precipitation of other metal hydroxides). The chelate is insoluble in all common organic solvents, thus the complex can not be extracted from the aqueous phase for the measurement of absorbance. The method is relatively insensitive and a large sample size has to be used for trace analysis.

Oxalydihydrazide with acetaldehyde gives a deep pink-colored complex with copper in the aqueous medium. It has a molar absorptivity comparable to the complex of dithizone. Unfortunately, the chelate formation is very sensitive to pH. The color is maximal over pH range of 8.4 - 9.1. Below pH 7.4 complex formation is negligible and at a pH value higher than 9.1, the intensity for the color is decreased. This reagent is thus not suitable for routine analysis because of its critical dependence on the pH.

1, 5-Diphenylcarbohydrazide[1,9] is probably the most sensitive reagent for determination of trace amount of copper. The copper chelate has a molar absorptivity of 158,000 in water which is more than ten times the molar absorptivity of the copper diethyldithiocarbamate in chloroform. The relationship between absorbance and concentration of the copper complex conforms to Beer's Law over the concentration range of 0.01 ppm. Cu(A = 0.010) to 0.26 ppm. Cu(A = 0.200). In spite of this extremely high sensitivity, this reagent has several disadvantages. The color development depends very much on the pH of the solution and no plateau is observed as shown in Figure X. An error of ± 0.01 pH units corresponds to an error of ± 2% in the determination. It is also noted that the color development reaction is a function of reaction time. The relationship between absorbance and the concentration of copper solution follows Beer's Law for only eight minutes. During this time interval, there is a gradual but constant increase in color intensity. Beyond that, the color intensity continues to increase, but the reaction becomes erratic. Thus the absorbance must be measured at a fixed time after the addition of the reagents. The reaction rate also depends on the temperature and the amount of reagent used. Figure XI shows the effect of temperature on reaction rate. In addition to this, the ethanolic reagent solution is unsuitable for analytical purposes. The reason is that it will be oxidized by air easily if it is exposed for more than an hour.

Stoner and Dasler[150] later suggested the copper 1, 5-diphenylcarbohydrazide to be extracted into benzene. This offers some advantages over the original method. Maximum color development in the test solution is almost instantaneous, the color intensity does not change over a twenty-four hour period, and the benzene solution of 1, 5-diphenylcarbohydrazide is stable for at least two weeks when stored in a borosilicate container. The disadvantages are that the molar absorptivity decreases to 55,000, i.e. it is just about one-third of that in water. The effect of pH on color development is still quite critical but there seems to be a small plateau over the pH range of 11 - 12. By using ^{64}Cu as a tracer in this study, the percentage of extraction of copper-64 1, 5-diphenylcarbohydrazide into benzene was determined as a function of the pH of the

FIGURE X[149]

Effect of pH on Color Development of Copper 1, 5-Diphenylcarbohydrazide.

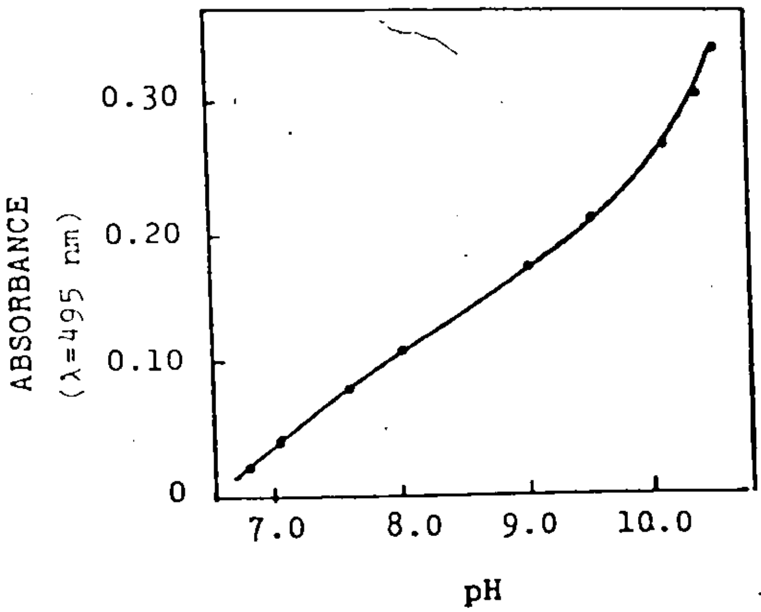
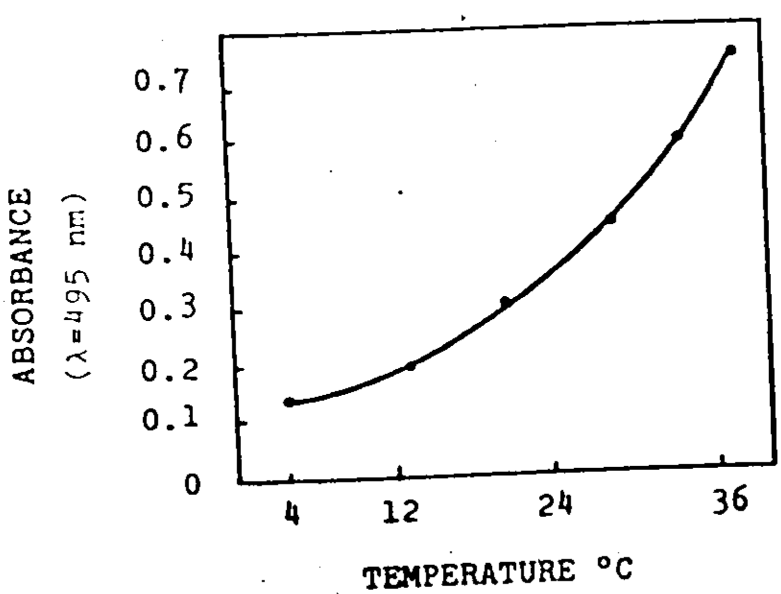


FIGURE XI[149]

Effect of Temperature on Reaction Rate of Copper 1, 5-Diphenylcarbohydrazide.

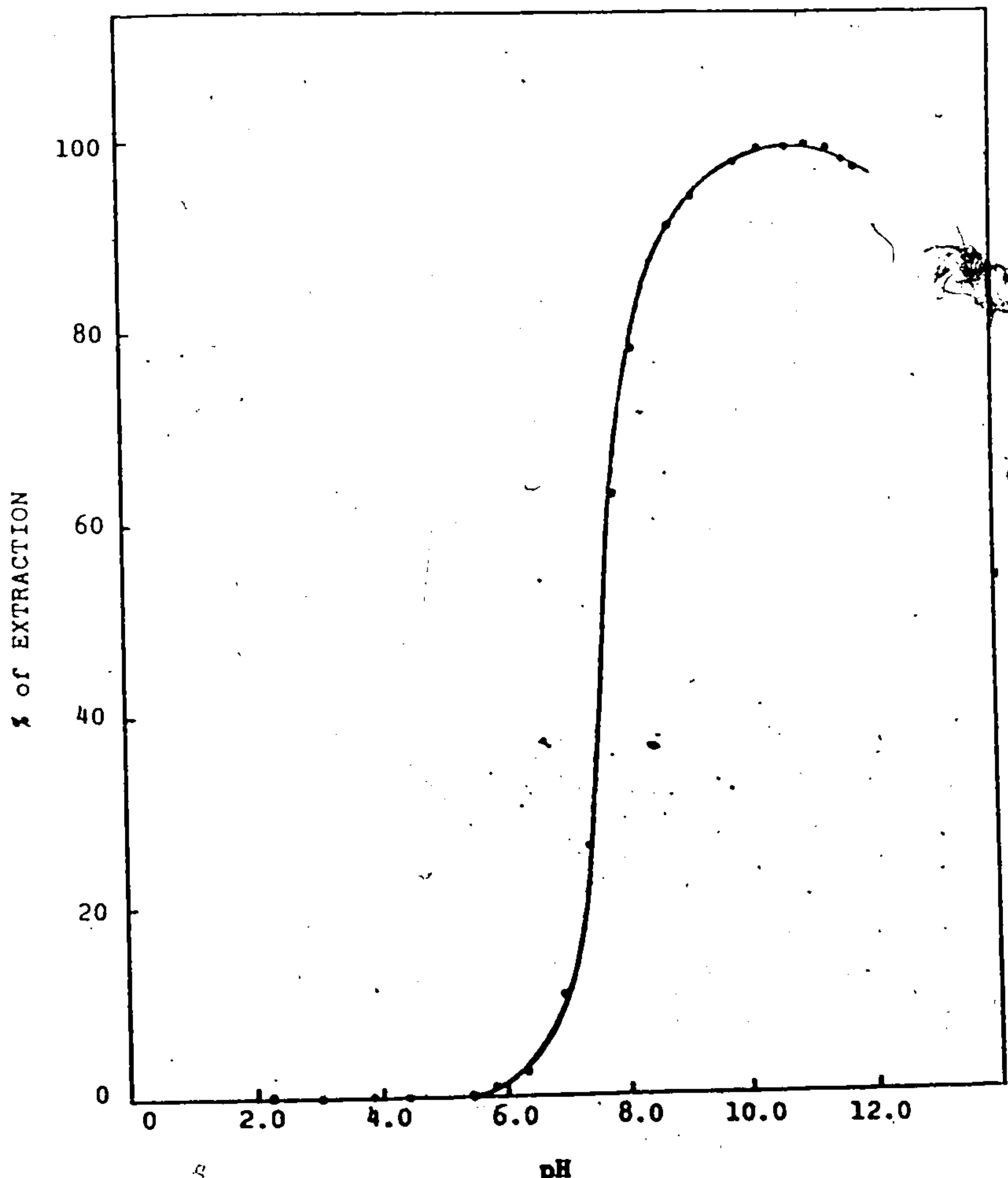


aqueous copper solution. Figure XII shows the result of the study. As can be seen from the figure, about 98% of the copper can be extracted into benzene over the pH range of 10.3 to 11.4.

Zinc dibenzyldithiocarbamate[133] is a reagent similar to the sodium salt. The copper dibenzyldithiocarbamate in benzene has an absorption maxima at 438 nm as shown in the absorption spectrum in Figure XIII. It has a molar absorptivity of 15,400 as determined from the standard calibration curve shown in Figure XIV. This reagent offers many advantages over its sodium salt and the other copper spectrophotometric reagents mentioned above. The reagent solution can be prepared easily by dissolving the salt in benzene. It is stable for at least two weeks. The yellow copper complex found in benzene is stable for at least one day. Quantitative transfer of copper into the benzene layer can be obtained with just one extraction step. The greatest advantage is that the extraction can be performed in an acidic medium. It was found in these studies that quantitative extraction of copper can be obtained from aqueous solutions of pH 7 to 6N HCl by using ^{64}Cu as a tracer. This differs from most of the other copper reagents (with the exception of dithizone) where extraction is carried out in basic medium. Thus neutralization with ammonia is not necessary and the addition of pyrophosphate can be avoided. The use of this reagent simplifies the procedure for determination of copper and the blank can easier be kept low. The use of benzene instead of carbon tetrachloride is preferred because

FIGURE XII

Effect of pH on Extraction of Copper 1, 5-Diphenylcarbohydrazide into Benzene.



% of EXTRACTION
100
80
60
40
20
0
0
2.0
4.0
6.0
8.0
10.0
12.0
pH

FIGURE XIII

Absorption Spectrum of Copper Dibenzyldithiocarbamate Complex in Benzene.

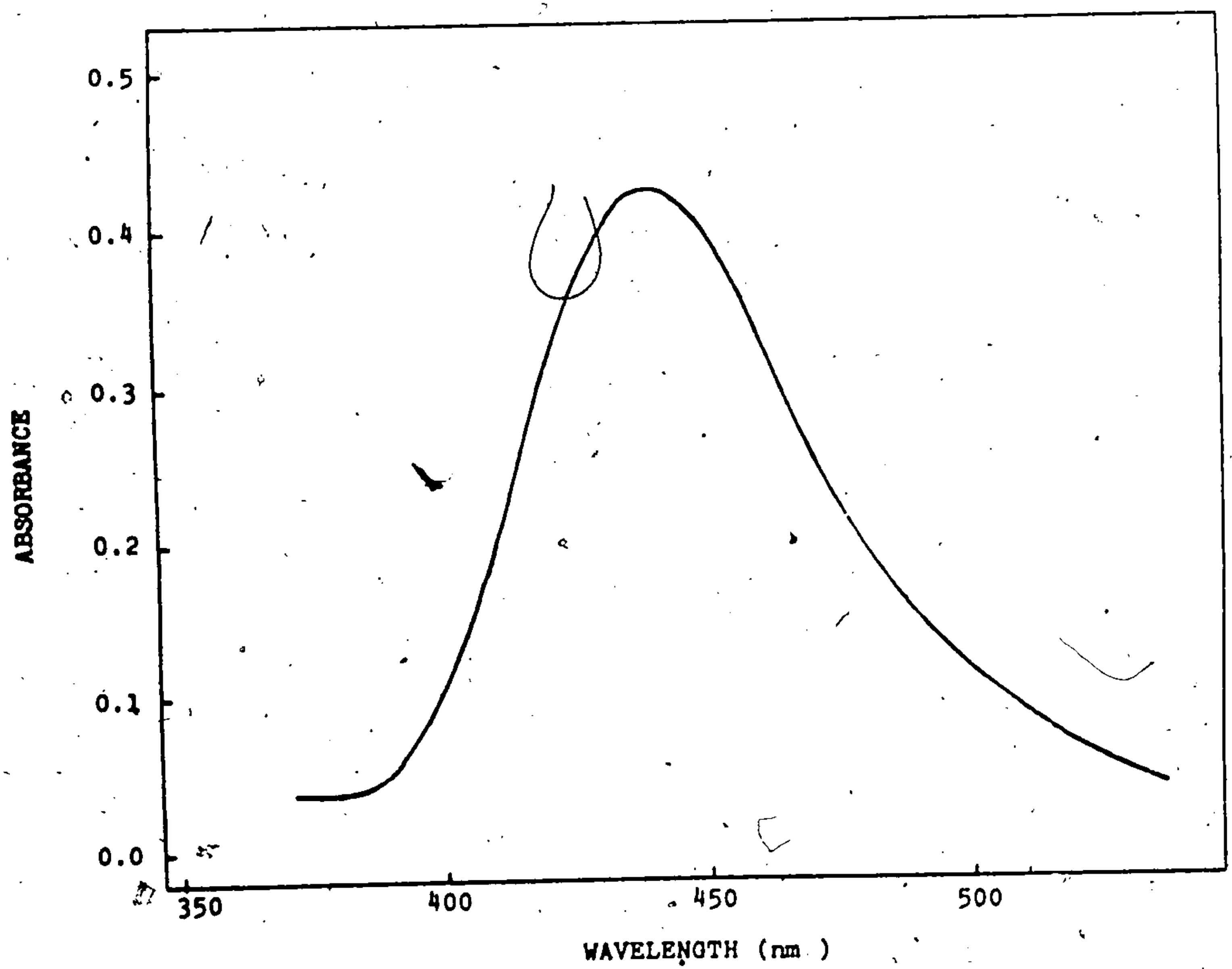

0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
350
400
450
500
WAVELENGTH (nm)

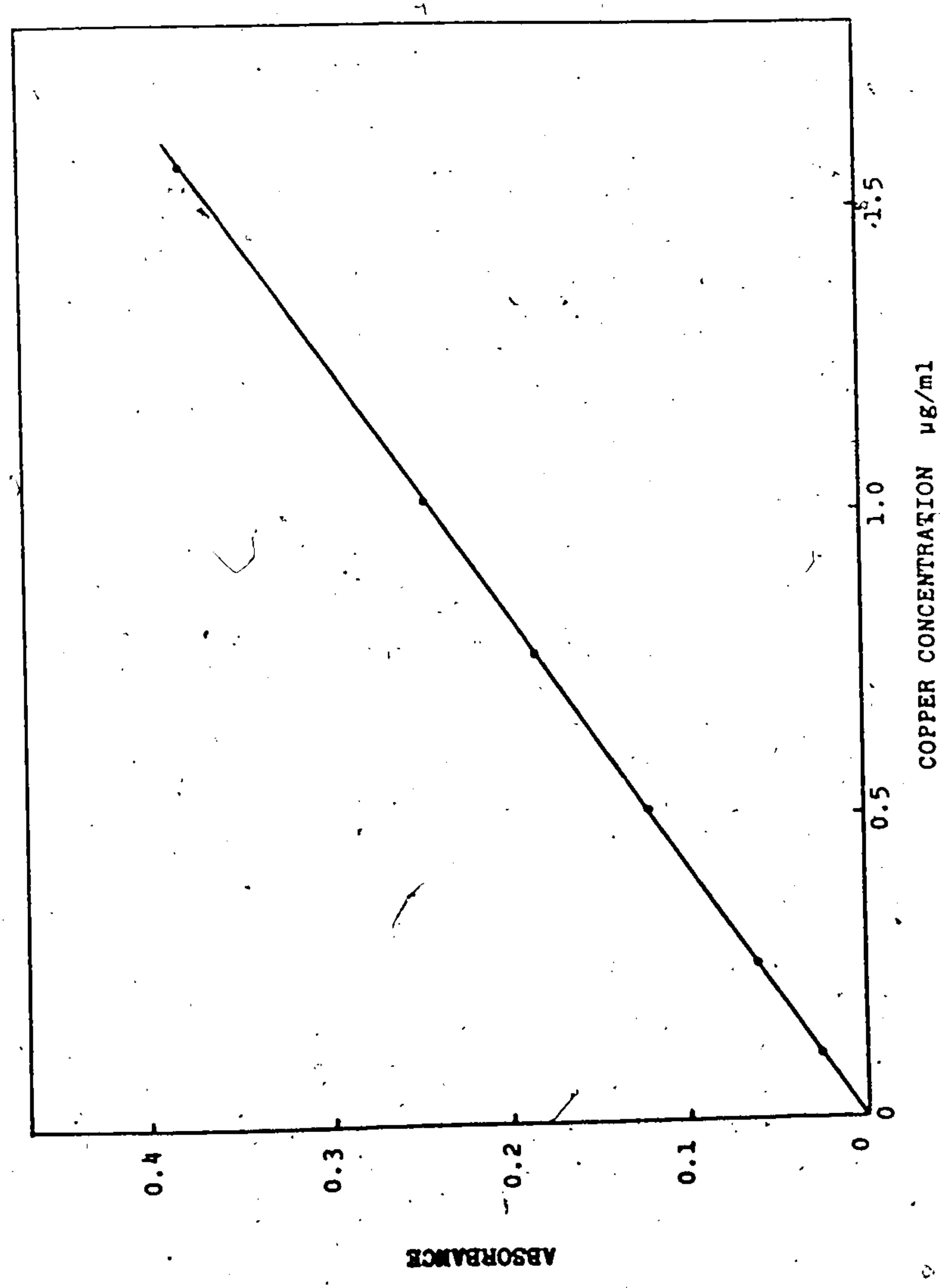
FIGURE XIV

Standard Calibration Curve for Copper Dibenzyldithiocarbamate in Benzene.

1.00 ml copper standard solution + 1.00 ml .3N HCl + 1.00 ml H_2O extracted by 1.00 ml 0.06% zinc dibenzyldithiocarbamate in benzene.

λ = 438 nm.

path length 10 mm.


ABSORBANCE
0.4
0.3
0.2
0.1
0
0
0.5
1.0
1.5
COPPER CONCENTRATION μg/ml

of the lower density of benzene compared to water. The benzene layer, being the top layer, can be easier transferred to the cuvette for the measurement of absorbance after the extraction in a polypropylene tube.

The effect of interference from other metallic ions was also studied. Different amounts of the metal halides were dissolved in 1M HCl solutions and the solutions were then extracted by 0.06% (w /v) zinc dibenzyldithiocarbamate in benzene. The absorbance of the benzene layer was then measured at 438 nm. No reading was obtained from the following ions: Sodium (10 mg), potassium (10 mg), magnesium (1.5 mg), calcium (1.5 mg), zinc (1.0 mg), nickel (<9 mg), cobalt (<6 mg), iron (III) (<2 mg), mercury (II) (<0.1 mg), antimony (III) (0.1 mg). The only interfering ion is bismuth, 50 μg gives an absorbance reading corresponding to 2 μg of copper. This interference from bismuth can be avoided by carrying out the extraction in strong hydrochloric acid solution. Bismuth is not easily extracted, while quantitative extraction of copper is still possible providing longer shaking time is used. Both mercury and antimony form colorless dibenzyldithiocarbamates which are extracted preferentially or along with the copper salt. The interferences may be eliminated by use of a larger amount of reagent which will extract mercury, antimony together with copper into benzene. In the analysis for copper in blood serum or whole blood, the ions which may cause interferences are present in such low concentration[176] that they cause no difficulty.

As a result, this reagent is very suitable to be used in the analysis for the non-ceruloplasmin bound copper and total copper in blood serum. Extraction of the copper complex can be carried out directly with the supernatant after precipitation of the serum proteins with hydrochloric acid and trichloroacetic acid, in the case of total copper determination. For the non-ceruloplasmin bound copper, the hydrochloric acid fraction collected from the Chelex 100 resin column can be used directly without further treatment. The procedures for the analyses have been described in section 2.6.3. and section 2.7.

A pooled sample of pig plasma was used to study this spectrophotometric method. It was analyzed simultaneously by neutron activation analysis and this spectrophotometric method. Table IX shows the results of these studies. Neutron activation analysis gives a result of 2.11 ± 0.05 ppm., whereas the spectrophotometric method gives 2.09 ± 0.04 ppm. By applying the t test,[189,190] t is found to be 0.803 which is less than the critical value of t at 5% probability level (2.228). It may be concluded that the two methods agree quite well with each other.

3.8. Comparison Between the Three Methods of Analyses.

In order to compare the three methods of analyses, two pooled pig plasma samples were analyzed for total copper

TABLE IX

Determination of Total Copper in a Pooled Pig Plasma Sample by Neutron Activation Analysis (NAA) and a Spectrophotometric Method Using Zinc Dibenzyldithiocarbamate as the Chelating Reagent.

Sample Size: 500 μl.

Copper Concentration μg/ml

NAA	Spectrophotometric Method
2.14	2.12
2.10	2.08
2.08	2.06
	2.02
	2.08
	2.14
	2.08
	2.08
	2.14
2.11 ± 0.05	2.09 ± 0.04

and non-ceruloplasmin bound copper repeatedly by neutron activation analysis, atomic absorption spectroscopy and the spectrophotometric method. Table X and Table XI give the results of these studies. As can be seen, they agree very well with each other. By applying the F test[138] to the results in Table XI, which has more data, it is found that the F values are 3.30 and 0.65 for the non-ceruloplasmin bound copper and total copper determination respectively. This is less than the critical value of F at 5% level (4.26). This indicates all the three methods proposed in these studies can be used successfully to determine the two forms of bound copper in blood serum.

In a comparison of the sensitivities of the three methods, neutron activation analysis has the highest sensitivity. The sensitivity for copper determination by neutron activation analysis is found to be 10^{-4} µg. In atomic absorption spectroscopy, the sensitivity is 0.03 µg/ml/1%. In the spectrophotometric method, the sensitivity is 0.004 µg/cm^2 for A = 0.001. Actually, 5×10^{-4} µg copper can be determined without difficulty by neutron activation analysis. In the other two methods, the concentration of copper solution is measured and the amount of copper is then calculated. In atomic absorption spectroscopy, 1.50 ml of methyl isobutyl ketone was found to be necessary to give a reliable signal for the atomic absorption spectrophotometer used in these studies. Thus 4.5×10^{-2} µg copper is required to give a 1% absorption in the flame. The detection limit is thus

TABLE X

Non-ceruloplasmin Bound Copper and Total Copper in Pig Plasma Using Three Different Analytical Methods for the Copper Determination (Pooled Sample No. 1).

Non-ceruloplasmin Bound Copper (µg/ml)

Sample Size: 1.00 ml.

NAA	AAS	Spectrophotometric Method
0.68	0.68	0.63
0.63	0.63	0.62
0.63		
0.65	0.65	0.62

Total Copper (µg/ml)

Sample Size: 0.50 ml.

NAA	AAS	Spectrophotometric Method
2.32	2.16	2.28
2.37	2.21	2.22
2.30	2.24	2.27
2.33	2.20	2.26

NAA - Neutron Activation Analysis

AAS - Atomic Absorption Spectroscopy

TABLE XI

Non-ceruloplasmin Bound Copper and Total Copper in Pig Plasma Using Three Different Analytical Methods for the Copper Determination (Pooled Sample No. 2).

Non-ceruloplasmin Bound Copper (μg/ml)

Sample Size: 1.00 ml.

NAA	AAS	Spectrophotometric Method
0.58	0.56	0.57
0.60	0.59	0.58
0.59	0.57	0.56
0.59	0.58	0.58
0.59	0.57	0.57

Total Copper (μg/ml)

Sample Size: 0.50 ml.

NAA	AAS	Spectrophotometric Method
1.76	1.76	1.72
1.79	1.78	1.74
1.74	1.76	1.74
1.73	1.70	1.76
1.76	1.75	1.74

NAA - Neutron Activation Analysis

AAS - Atomic Absorption Spectroscopy

approximately 2×10^{-2} μg. (A = 0.002). A much lower detection limit can be obtained by using the graphite cell atomic absorption spectroscopy[1,2] and a smaller size of sample solution. In the spectrophotometric method, a minimum absorbance reading of 0.005 is required to obtain reliable results with the instrument used. This increases the minimum amount of copper measureable to 2×10^{-2} μg. The detection limit can be lowered by using the method of precision colorimetry[1,3] with the Beckman model DU Spectrometer or similar instruments, but this would introduce error in the absorbance readings and lower the precision and accuracy of the analyses. In these studies, accurate and precise analyses are the aims rather than the detection of smallest amounts of copper. Moreover, the copper concentration in serum falls well above the detection limits of the three suggested methods. Thus the method of precision colorimetry and graphite cell atomic absorption spectroscopy were not used.

Neutron activation analysis also has the advantage over the other two methods in that there is less possibility for contamination by outside copper. The samples were only freeze-dried and then irradiated. No other handling or addition of reagents is required. Contamination by inactive copper after irradiation would not affect the analysis. In the case of the other two methods, the possibilities of contamination by the reagents and other sources do exist. The reagent blanks have to be determined to correct for it.

In neutron activation analysis neutron flux difference between sample and standard, counting statistics and interferences from other nuclear reactions would contribute errors. Since the sample is at the bottom of the polyethylene bottle and the standard is a small piece of aluminum sheet attached to the outside surface of the bottom of the bottle, the flux difference is negligible. The ^{64}Cu was isolated before counting and usually more than 5000 counts were accumulated. The error introduced here is about 1-2%. The only interference from other nuclides comes from the (n, p) reaction on ^{64}Zn. By irradiating some high purity zinc under the same irradiating conditions as in the determination of copper in serum, it was found that 1 μg of Zn would produce the same amount of ^{64}Cu as from 0.0014 μg Cu. The concentration of zinc in human blood serum is about 1.6 ppm.[176] Thus for one gram of blood serum, this amount of zinc (1.6 μg) would produce the same amount of ^{64}Cu as from 0.0022 μg copper. The error caused by this factor is negligible in the total coppper determination because of the comparatively large total copper concentration in blood serum (∿1 ppm.). In the case of the non-ceruloplasmin copper determination, the maximum error would be 4% even if we assume that all the zinc in blood serum would end up in the non-ceruloplasmin copper fraction. (The concentration of this copper is ∿ 0.06 ppm. section 3.9.1. and 3.9.2.). Thus it is reasonable to estimate the error in neutron activation analysis to be about 5%.

In the atomic absorption spectroscopic method errors may arise from incomplete liberation of copper from the proteins by hydrochloric acid and trichloroacetic acid, incomplete extraction of the copper complex into the organic layer, contamination by reagents and fluctuation of the absorbance reading. The first two factors have been discussed in section 3.5. The liberation of copper and the extraction of it into the organic layer were quantitive and reproducible. The contamination by reagents can be prevented by using high purity reagents and running a blank in parallel to the sample. The fluctuation of the absorbance reading depends largely on the stability of the instrument. An absorbance reading of ± 0.001 would give an uncertainty of copper concentration ± 0.007 μg / ml MIBK. The absorbance reading for the non-ceruloplasmin bound copper determination is approximately 0.02. Moreover due to the small volume of solvent used, only three to four readings can be recorded with the 0.1 second integrator for the sample or standard. Thus an error of ± 5% may be introduced by this factor alone. The use of Eppendorf microlitre pipettes to deliver the reagent solutions would also introduce an error of 1 - 2% in the measurement of volumes.

The situation is almost the same with the spectrophotometric method, except that the instrument is more stable. On the other hand, digital readout is not available with the instrument. An inherent absorbance reading of ± 0.002 would give an uncertainty of ± 0.01 μg/ml in the copper concentration, the same magnitude as in the atomic absorption spectroscopic method.

Finally, although neutron activation analysis has the great advantage of high precision and accuracy, it has its own disadvantages. A high flux neutron source and sophisicated radiation detection instruments are required. Specially trained personnel are also required. The comparatively simple and inexpensive instruments used for the analyses and the speed of analyses are certainly advantages for the methods of atomic absorption spectroscopy and spectrophotometry. These together with their reasonable accuracy and precision enable these two methods to be widely used for the copper analysis.

3.9. Copper Concentration in Human Blood Serum.

After the method for the determination of non-ceruloplasmin bound copper and total copper had been established, it was used to determine the copper concentration in human blood serum. Neutron activation analysis was mainly used in the following studies due to its high accuracy and precision. The easy access to the McMaster Nuclear Reactor and the availability of many radioactivity detection instruments were the other reasons for this choice. Blood was obtained in the morning before breakfast from male volunteers. That female subjects were not preferred at the beginning is due to the fluctuation of copper concentration in blood serum under different physiological conditions, (i.e. during pregnancy[23] or oral contraception[184,185] etc.). The results shown in the following sections are the averages of duplicate analyses for both the total copper and the non-ceruloplasmin bound copper, unless specified.

3.9.1. Caucasians.

The subjects for this group were ten Caucasians of age 20 to 30. The results are shown in Table XII. The total copper concentration varies from 0.890 to 1.180 ppm. with an average of 0.982 ppm. The non-ceruloplasmin bound copper concentration varies from 0.052 to 0.069 ppm. with an average of 0.062 ppm. From this data, the concentration of ceruloplasmin bound copper is calculated to be 0.920 ± 0.091 ppm. The concentration of ceruloplasmin is then estimated to be 27 mg/100 ml.

Table XII also shows the coefficients of variation for the two forms of bound copper of the ten subjects are almost the same (∿ 10%). Moreover, the percentage of non-ceruloplasmin bound copper in terms of total copper is not constant. It ranges from 5.59 to 7.26% (an average of 6.30%).

3.9.2. Chinese.

The blood serum of one Chinese male student (T.K.) was also used for analysis for copper. The total copper concentration was found to be 0.727 ppm. and the non-ceruloplasmin bound copper was 0.065 ppm. By comparing this with that of the caucasians mentioned in the previous

TABLE XII

Non-ceruloplasmin Bound Copper and Total Copper Concentration in Caucasian's Blood Serum.

Copper Concentration ppm.

Subject	Total Cu	NcCu	CCu	NcCu / Total Cu %
R.R.	0.916	0.065	0.851	7.10
M.T.	0.927	0.067	0.860	7.23
S.C.	1.180	0.066	1.114	5.59
H.F.	0.950	0.060	0.890	6.32
F.G.	0.950	0.069	0.881	7.26
G.N.	0.890	0.054	0.836	6.07
S.K.	0.911	0.052	0.859	5.71
C.W.	1.086	0.063	1.023	5.80
F.J.	1.050	0.066	0.984	6.29
S.B.	0.956	0.054	0.902	5.65
	0.982 ± 0.093	0.062 ± 0.006	0.920 ± 0.091	6.30

NcCu - Non-ceruloplasmin bound copper

CCu - Ceruloplasmin bound copper

section, the very low total copper concentration but comparable non-ceruloplasmin bound copper concentration is quite interesting. In order to know whether this is a special case or not, blood sera from nine other Chinese male students were also analyzed. They are of age between twenty and thirty. Most of them came from Hong Kong and have been in Canada for more than one year. Several of them have been living here for more than ten years. The results for these analyses are shown in Table XIII. In contrast to the Caucasians, the total copper concentration varies from 0.727 to 0.923 ppm. with an average of 0.792 ppm., non-ceruloplasmin bound copper varies from 0.053 to 0.070 ppm. with an average of 0.062 ppm. The ceruloplasmin bound copper is then calculated to be 0.730 ppm. and the ceruloplasmin is estimated to be 21.5 mg/100 ml.

Several interesting points can be pointed out by comparing Table XII and Table XIII.

1. There is no difference in the averages of the concentration of non-ceruloplasmin bound copper between the two groups. Moreover the ranges are almost the same (0.052 - 0.069 ppm. in the Caucasians and 0.053 - 0.070 ppm. in the Chinese). The coefficients of variation are also similar (9.7% and 8.1% respectively).

2. There is a significant difference ($p < 0.01$) in the averages of the total copper concentration between the two groups. The copper concentration in Caucasians, is

TABLE XIII

Non-ceruloplasmin Bound Copper and Total Copper Concentration in Chinese' Blood Serum.

Copper Concentration ppm.

Subject	Total Cu	NcCu	CCu	$\frac{\text{NcCu}}{\text{Total Cu}}$ %
C.E.	0.744	0.062	0.682	8.33
N.P.	0.793	0.070	0.723	8.83
C.L.	0.923	0.066	0.857	8.15
L.H.	0.785	0.054	0.731	6.88
T.C.	0.769	0.053	0.716	6.89
L.C.	0.772	0.063	0.709	8.16
$T.R_1$.	0.728	0.059	0.669	8.10
$T.R_2$.	0.812	0.064	0.748	7.88
L.P.	0.862	0.065	0.797	7.54
T.K.	0.727	0.065	0.662	8.94
	0.792 ± 0.062	0.062 ± 0.005	0.730 ± 0.060	7.87

NcCu - Non-ceruloplasmin bound copper

CCu - Ceruloplasmin bound copper

~ 24% higher than that in Chinese. This can be attributed to a higher concentration of the ceruloplasmin bound copper in the former group (0.920 and 0.731 ppm. respectively). In fact, there is only one person (C.L.) in the Chinese group who has a ceruloplasmin bound copper concentration (0.857 ppm.) which just falls within the range of the Caucasians. All the other Chinese have a much lower ceruloplasmin bound copper concentration than the Caucasians. Similar results were also observed by Shokeir.[188] He determined the serum ceruloplasmin levels in several ethnic groups and found that Orientals have a lower level of ceruloplasmin than either Negroes or Caucasians, whereas the level in Amerindians is higher. Shokeir concluded that as far as diet, climate and state of health are concerned, no significant differences were observed between the groups under investigation. Thus racial differences seem to play a more important role than the influence of environment. A genetic basis may be favored for the variations. More detailed studies are required to justify this idea.

3. A constant percentage of non-ceruloplasmin bound copper in terms of total copper is not observed in either groups.

3.9.3. Variation as a Function of Time.

The variation of the concentrations of the total copper and non-ceruloplasmin bound copper in human blood serum as a function of time was studied. The subject was

Mr. T. K. The study consists of two parts. Firstly, the fluctuation was studied during the year of 1972. Blood was obtained from the subject every two weeks on Tuesday morning at ten o'clock. Duplicate analyses of the samples were carried out using neutron activation analysis for the copper determination. Figure XV shows the results of the studies. The average concentration of the total copper is found to be 0.727 ppm. with a standard deviation of 0.005 ppm., whereas the non-ceruloplasmin bound copper is 0.065 ± 0.001 ppm. If the standard deviation is recalculated by using each individual result of the analyses instead of the averages of the duplicate analyses of each sample, then it is 0.007 ppm. and 0.002 ppm. for the total copper and non-ceruloplasmin bound copper determinations respectively. The relative error (∿1% and 3%) is the same as in the analyses of the pooled human serum (Table VI). Thus, it is concluded that the variations in the results are due to the experimental errors rather than physiological changes with time. The concentrations of the two forms of copper remain remarkably constant and do not vary with time during the period of one year.

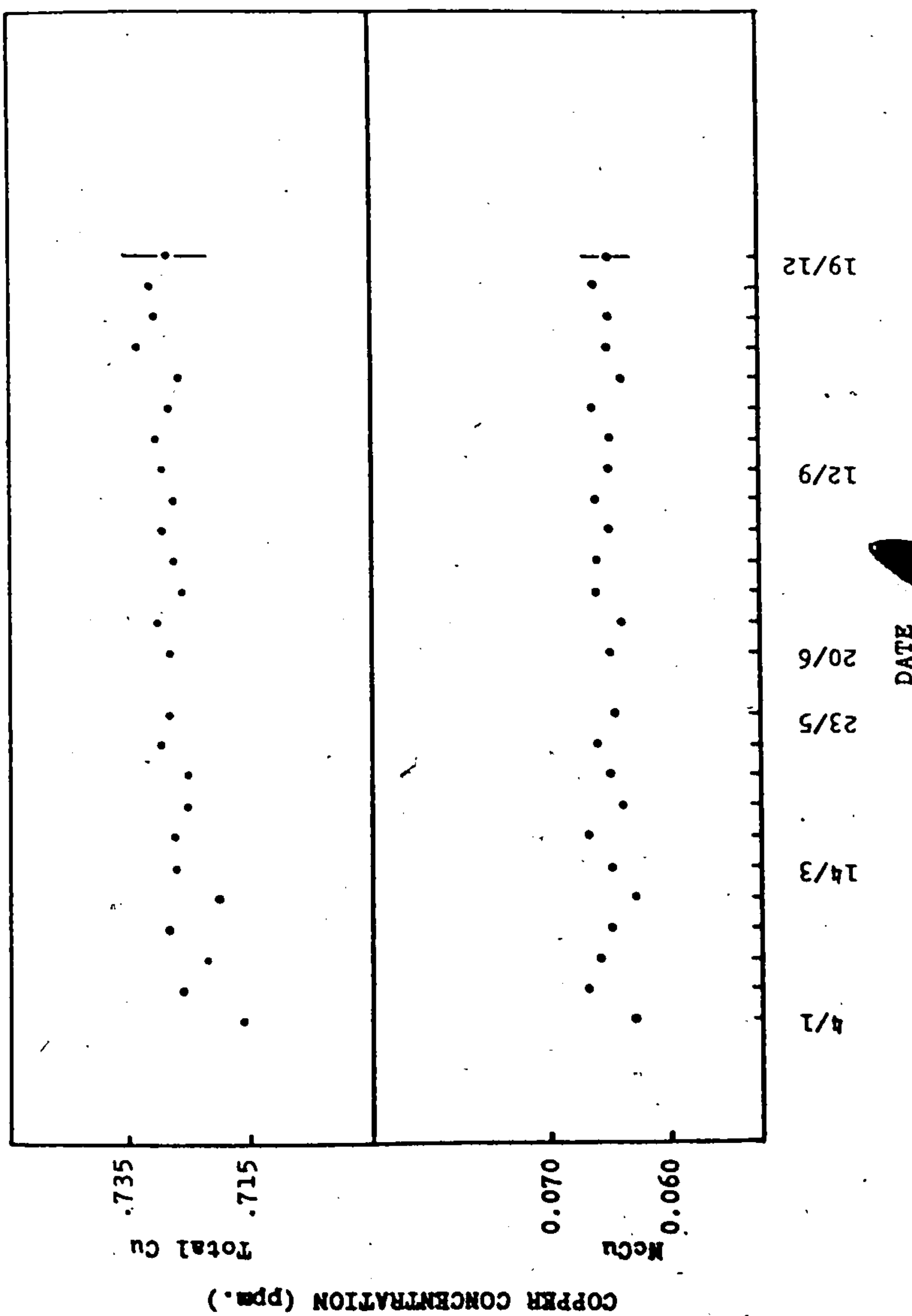
Secondly, the circadian variation within one day was also studied. Lifschitz and Henkin[117] recently reported that there is a circadian variation in total serum copper concentration in man. It is greater than the 24 hr mean at 10:00 a.m. and 2:00 a.m., is essentially equal to the mean at

FIGURE XV

Concentration of Copper in the Blood Serum of a Chinese as a Function of Time During One Year of Studies.

NcCu - Non-ceruloplasmin bound copper

The ranges for one of the analyses are also shown by the bars above and below the averages.


COPPER CONCENTRATION (ppm.)
Total Cu
.735
.715
NcCu
0.070
0.060
DATE
4/1
14/3
23/5
20/6
12/9
19/12

6:00 p.m. and 10:00 p.m., and falls to a level significantly below the mean at 2:00 a.m. and 6:00 a.m. Serum concentration of ceruloplasmin shows a similar but less marked circadian pattern of variation. The great increase (~14% referred to the mean) during the four hours period of 6:00 a.m. to 10:00 a.m. is most interesting. Experiments were carried out to study the phenomenon. Blood serum was obtained from Mr. T. K. at 6:00 a.m., 10:00 a.m. and 6:00 p.m. of one day. Duplicate analyses were done for the non-ceruloplasmin bound copper, whereas triplicate analyses were carried out for the total copper determination.

Table XIV shows the results of this study. Figure XVIa and Figure XVIb are graphic plots of these results on the same concentration scale as in Figure XV. By comparing these three figures, it can be seen clearly that there are variations in the concentrations of both total copper and non-ceruloplasmin bound copper at different times of one day in constrast to the constancy at the same time of the day throughout one year. When the lines joining the points at 10:00 a.m. and 6:00 p.m. are extrapolated to intercept at the 6:00 a.m. level, the concentrations of both forms of copper decrease back to the concentration of 24 hours ago within experimental errors as shown by the dot lines in the figures. Thus the variations are definitely due to physiological changes with time instead of experimental errors. Moreover, the variations of the two forms of bound copper are almost

TABLE XIV

Concentration of Copper in the Blood Serum of a Chinese as a Function of Time in One Day of Studies.

Cu Concentration ppm.	Time 6:00 a.m.	10:00 a.m.	6:00 p.m.
Total Cu	0.650	0.725	0.692
	0.656	0.708	0.687
	0.672	0.730	0.675
	0.659	0.721	0.685
NcCu	0.054	0.065	0.060
	0.058	0.068	0.064
	0.056	0.066	0.062
CCu	0.603	0.655	0.623

NcCu - Non-ceruloplasmin bound copper.

CCu - Ceruloplasmin bound copper, the average difference between total Cu and NcCu.

FIGURE XVIa

Concentration of Copper in the Blood Serum of a Chinese as a Function of Time in One Day of Studies.

The ranges for the triplicate determinations are shown by the bars above and below the averages.

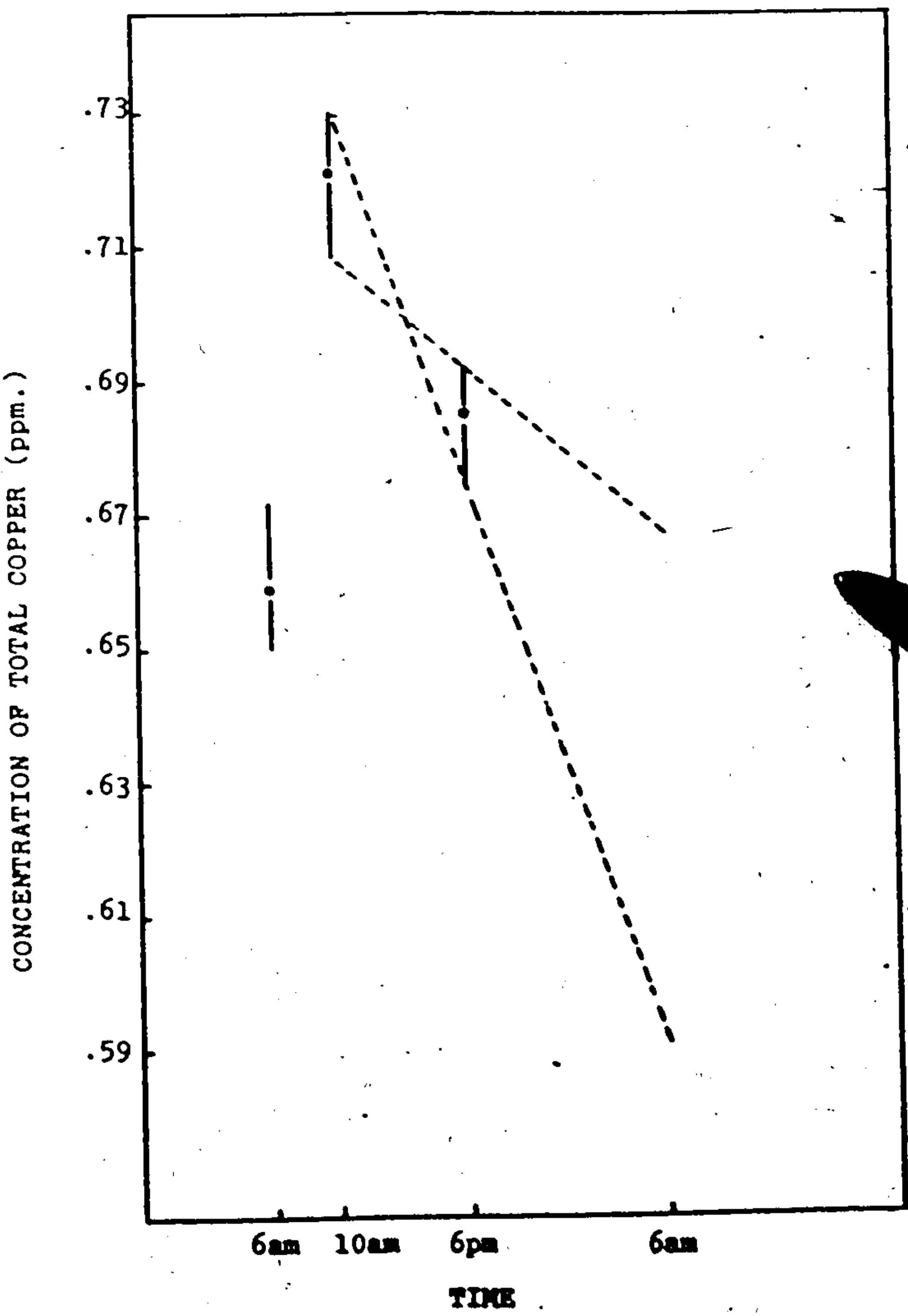
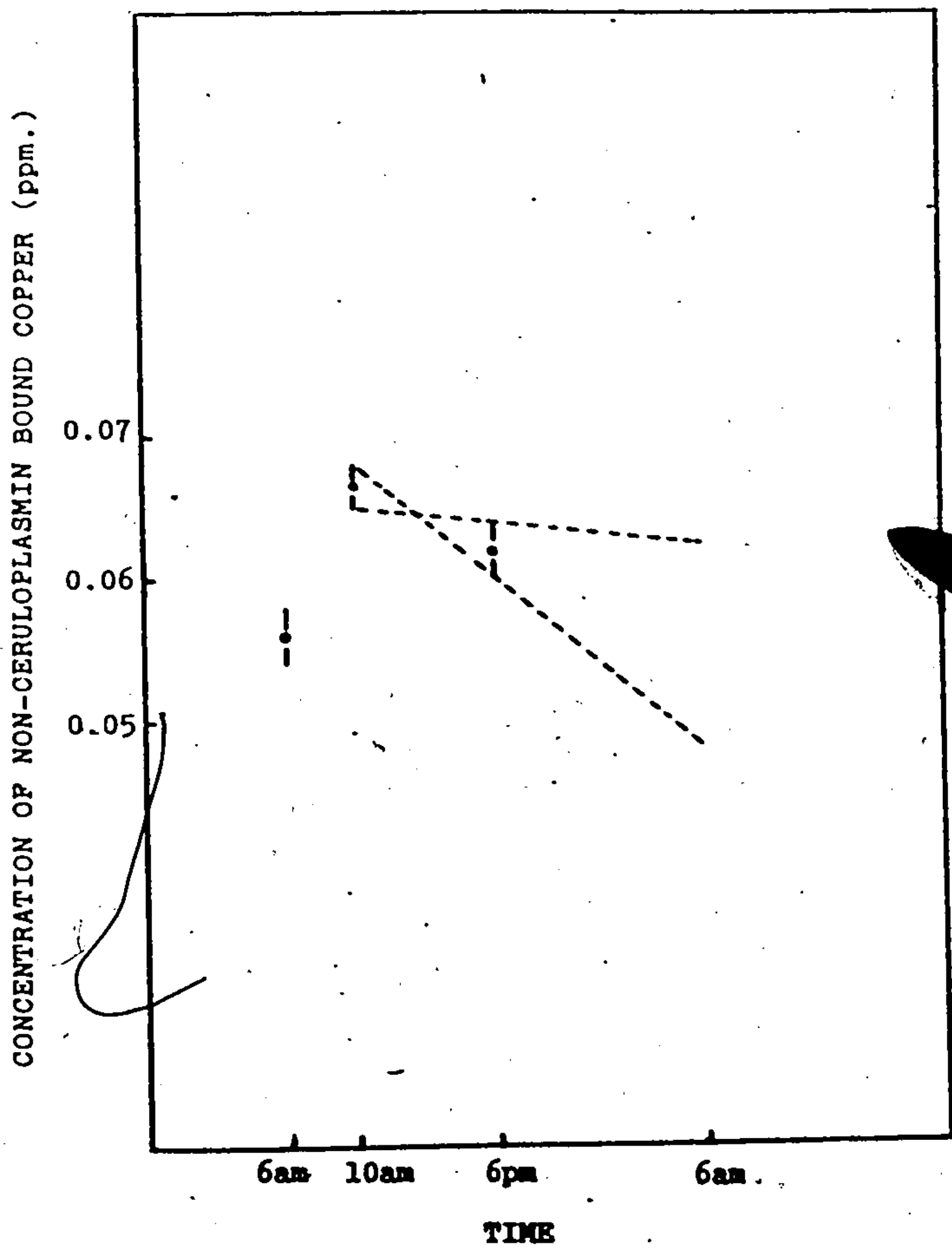

CONCENTRATION OF TOTAL COPPER (ppm.)
.73
.71
.69
.67
.65
.63
.61
.59
6am
10am
6pm
6am
TIME

FIGURE XVIb

Concentration of Copper in the Blood Serum of a Chinese as a Function of Time in One Day of Studies.

The ranges for the duplicate determinations are shown by the bars above and below the averages.


CONCENTRATION OF NON-CERULOPLASMIN BOUND COPPER (ppm.)
0.07
0.06
0.05
6am
10am
6pm
6am
TIME

"parallel" to each other. The ratio of the ceruloplasmin bound copper to non-ceruloplasmin bound copper are 10.8, 9.9 and 10.0 at the different times. (This is constant within experimental errors.) These results show that the circadian variation of total copper concentration is due to the variations not only in ceruloplasmin bound copper, but also in the non-ceruloplasmin bound copper. The latter factor could not be concluded from the studies by Lifschitz and Henkin[117].

3.9.4. A Pathological Case.

The blood plasma of a person with glomerulonephritis was analyzed for total copper and non-ceruloplasmin bound copper. This person has been for two years on home hemodialysis three times a week. Blood was obtained just before and after the overnight dialyzing process. Triplicate analyses on each sample were carried out. Table XV shows the results of this study. Before dialysis, the copper concentration was found to be 1.06 ± 0.02 ppm. for total copper and 0.070 ± 0.0025 ppm. for the non-ceruloplasmin bound copper. After dialysis they are 1.09 ± 0.02 ppm. and 0.079 ± 0.0025 ppm. respectively. By applying the t test, the increase is not significant for the total copper. Inspite of this statistical consideration, an inspection of Table XV shows that there is only a small overlap of the two extreme values (1.08 and 1.07 ppm. respectively).

In the case of the non-ceruloplasmin bound copper, the t value is 4.93 which is much greater than the critical

TABLE XV

Concentration of Copper in Blood Plasma of a Person with Glomerulonephritis Before and After the Overnight Dialyzing Process.

	Before Dialysis	After Dialysis
Total Cu (ppm.)	1.08	1.09
	1.04	1.11
	1.05	1.07
	1.06	1.09
NcCu (ppm.)	0.072	0.076
	0.070	0.081
	0.067	0.079
	0.070	0.079
CCu (ppm.)	0.99	1.01

NcCu - Non-ceruloplasmin bound copper

CCu - Ceruloplasmin bound copper, the average difference between total Cu and NcCu.

value of t at 5% level (t = 2.78). This shows that the increase is significant. Furthermore, the big difference in the averages (0.009 ppm.) and the difference between the low and high values (0.011 ppm.) compared to that of the analyses of the pooled sample shown in Table VI also indicates the increase of non-ceruloplasmin bound copper is a real one. These results suggest that there is some transfer of copper from the dialysate to the blood stream. In fact, if the dialysate were copper free, a reduction of the non-ceruloplasmin bound copper could be expected. By analyzing for copper in the dialysate with atomic absorption spectroscopy, it is found that the concentration of copper is approximately 15 ppb. This dialysate solution was diluted with deionized water in the ratio of 1:35 during the dialyzing process at a flow rate of 300 ml/min for nine hours. As a result, ~100 μg Cu was involved in the process. Table XV shows that the non-ceruloplasmin bound copper increased by 0.009 ppm. after dialysis. This corresponds to an increase of ~27 μg Cu in the total plasma by estimating the blood plasma volume of the person to be ~3 l. This increase seems to be somewhat high because copper administered intravenously was found to disappear very rapidly from the blood ($T_{1/2}$ <10 min.)[20]. Nevertheless, this experiment indicates that there is a transfer of copper from the dialysate and it seems likely that all ~100 μg will enter the blood stream of the user. This is strongly aided by the complexing of the copper by albumin and thus establishing a strong positive gradient into the blood. Obviously some of the transferred copper will be used in the synthesis of ceruloplasmin. This would cause a slight increase in the concentration of ceruloplasmin bound copper as indicated in Table XV. The remaining fraction would end up in other tissues of the body.

Finally, comparing the total copper concentration of the blood plasma of this person with that of the ten subjects in the Caucasian group (Table XII), there is no significant difference, but the non-ceruloplasmin bound copper of this person is definitely higher than the others ($P < 0.01$).

The foregoing discussion warrants further study as trace element poisoning has recently became a concern of nephrologists. It would also be interesting to study patients just before they go on dialysis and possible changes after the treatment has started. This is justified by the observation that people with nephritis have elevated copper level[191].

CONCLUSIONS

This section relates the contents of this thesis to the points raised in DELINEATION OF THE PROBLEM. Obviously not all the topics raised in the latter chapter could be investigated. Only these were dealt with in varying degrees of detail.

(1) A method was developed for the separation of ceruloplasmin copper from non-ceruloplasmin copper using ~3 ml blood serum.

(2) The determination on non-ceruloplasmin copper (~0.15 μg Cu) carries an error of ± 4% (1 σ, assuming a Gaussian distribution). The total copper determination (requiring 0.5 ml = 0.5 μg Cu) has a standard deviation of ± 2%. The latter appears to decrease as experience is gained by the individual experimenter (see FIGURE XV). The concentration of ceruloplasmin copper can be calculated accurately from the difference of **total** copper and non-ceruloplasmin copper.

(3) Neutron-activation analysis, atomic-absorption spectrometry and a spectrophotometric method using zinc dibenzyldithiocarbamate give the same results for the copper determinations.

(4) The analysis of the serum from ten Caucasians and ten Chinese demonstrates that the latter group has levels lower by ~24%. The probability that this is not the case, i.e. the two groups have identical ceruloplasmin concentration,

is 1 in 10^4. There is no difference in the non-ceruloplasmin copper concentration implying a genetic effect in regards to the apo-ceruloplasmin production.

(5) The concentration of both copper fractions (as measured in one individual over a period of one year) is remarkably constant. In fact, no changes were found within the error of the analytical method. This indicates a hemostatic control within ~1%. It is essential to collect blood sample at the same time of day for this type of study.

(6) The circadian variation of ceruloplasmin levels in human serum was confirmed.

(7) Although only one short-term experiment was done the indications are strong that the non-ceruloplasmin copper follows the pattern of the ceruloplasmin during the circadian cycle; i.e. the ratio of ceruloplasmin copper over non-ceruloplasmin copper is constant. That is interesting because the same ratio is not constant for Caucasians in relation to Chinese.

(8) Based on the analysis of only one person on hemodialysis there appears to occur a definite increase in the non-ceruloplasmin copper which can easily be rationalized as up-take of copper from the dialysate. The ceruloplasmin level was found to be normal in terms of the so-called normal range. The blood of patients with glomerulonephritis- but not on dialysis - have elevated concentrations.

Concerning the biosynthesis of ceruloplasmin some interesting speculations come to mind. The production of the protein part is obviously governed by the genetic code. Equally obvious is that there is no code for the incorporation of copper. The synthesis of ceruloplasmin could proceed in two steps: (a) the protein production, (b) the incorporation of copper. (b) has to follow (a) immediately and leads to the particular secondary and/or tertiary structure. The kinetics of this reaction should be interesting as the collision probability for eight copper ions "hitting" concurrently the exactly specified sites must be essentially nil. Assuming a sequence of copper-ion incorporation might mean a slow last step as by this time most of the secondary and tertiary structures should be established to some extent. Furthermore, the fact that four copper ions are in the 2^+ state and the other four in the 1^+ state renders the situation even more complicated and the question also arises "who looks after" the required reduction of Cu^{2+} which is normally present. Finally, as there exists a circadian variation in the synthesis of ceruloplasmin there could either be a "clock" governing this process or the ceruloplasmin production is the consequence of the non-ceruloplasmin concentrations. This would mean that the circadian variation of the later is the primary event.

APPENDIX

<u>In Vivo Addition of Copper-64 to Rabbit Blood Serum</u>.

The *in vivo* addition of ^{64}Cu to rabbit blood serum was also studied. The techniques and experimental procedures used were similar to those for the rat as described earlier. Blood was drawn twenty-four hours after the injection of carrier-free ^{64}Cu. Protein fractionation on the Bio-Gel P-150 column is shown in Figure XVI. The curve for the ^{64}Cu radioactivity shows a striking difference with that for a rat. (The radioactivity curves are the same for both the rat and the rabbit in the *in vitro* studies. It consists of a single peak coincides with the albumin.) It consists of two semi-separable peaks. The first one corresponds to the ceruloplasmin bound copper, whereas the second one corresponds to the copper bound to albumin. Upon resolving it into two Gaussian-shaped curves, with the DuPont 310 Curve Resolver, it is found that 53% of the ^{64}Cu activity is contributed from the ceruloplasmin bound copper, while the other 47% comes from the albumin bound copper.

As to the separation of the non-ceruloplasmin bound copper from ceruloplasmin bound copper on the Chelex 100 resin column, 51.7% of the ^{64}Cu activity was found in the ceruloplasmin fraction (average of duplicate columns, 51.4% and 52.0%). This agrees with that of gel-chromatography within experimental errors.

FIGURE XVII

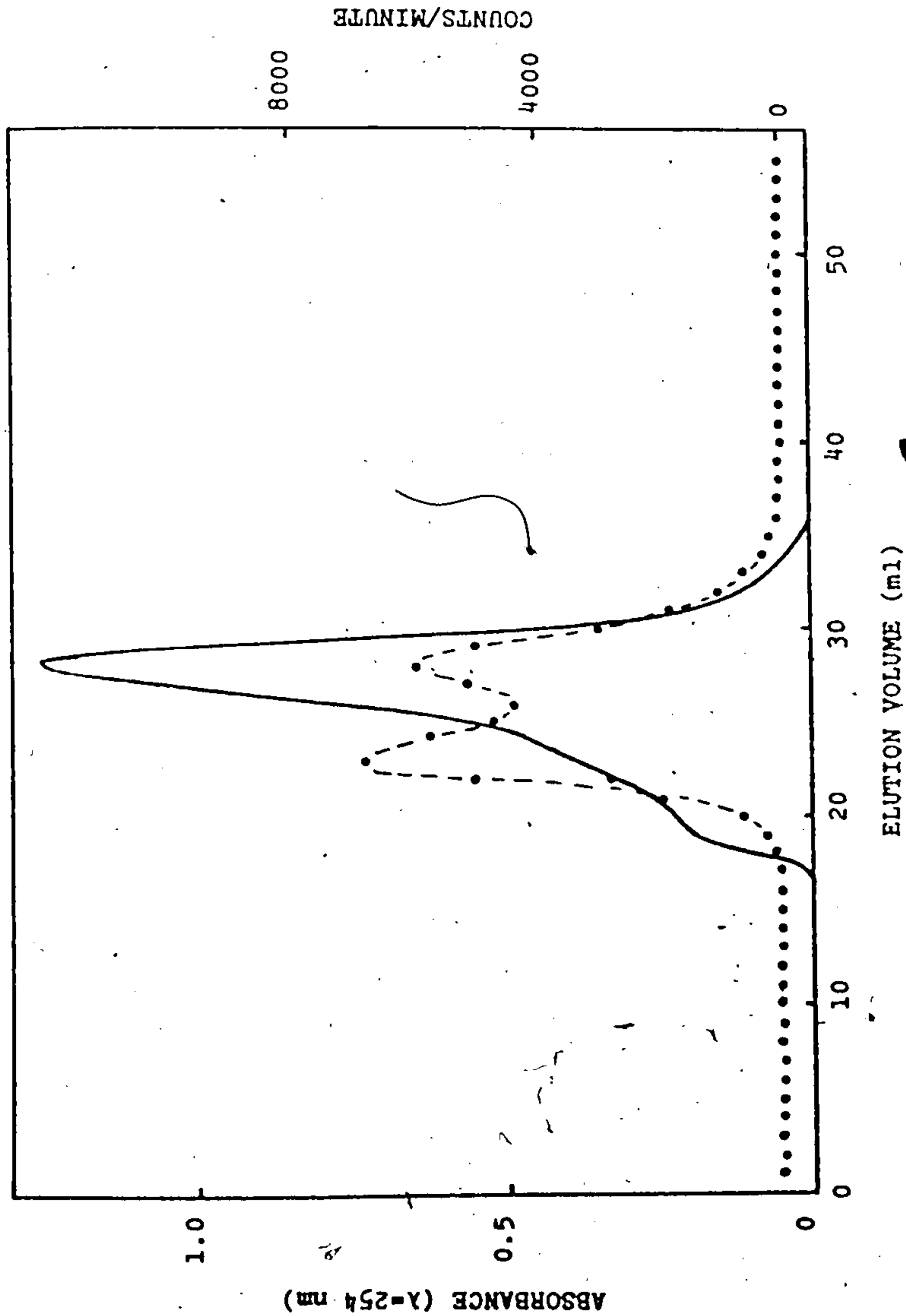
Bio-Gel P-150 Chromatography of _In Vivo_ ^{64}Cu Labelled Rabbit Blood Serum.

Sample Size: ~1.0 ml.

Column Size: 1.6 x 35 cm.

Protein Distribution Curve - Solid Line

^{64}Cu Activity Curve - Dot Line


ELUTION VOLUME (ml)
0
10
20
30
40
50
ABSORBANCE (λ=254 nm)
0
0.5
1.0
COUNTS/MINUTE
0
4000
8000

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