

IDENTIFICATION OF METAL PROTEIN COMPLEXES

THE COMBINATION OF RADIOANALYTICAL TECHNIQUES
WITH GEL CHROMATOGRAPHY FOR THE IDENTIFICATION
OF METAL-PROTEIN COMPLEXES

By

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SCOPE AND CONTENTS:

A new technique for the systematic identification of metal-protein complexes combining gel chromatography with either neutron activation analysis or radioactive tracer methods has been proposed. The technique has been tested on the copper in serum situation to evaluate the results obtained on a well-known system.

It was then applied to manganese in serum, manganese in erythrocytes and copper in erythrocytes. The results indicate that serum contains two manganese-binding proteins, one of low molecular weight and relatively labile in nature, the other of higher molecular weight and incorporating radioactive manganese in vivo at some definite time interval subsequent to the isotope's administration.

Manganese in erythrocytes occurs as a porphyrin bound to apoglobin as a manganese analogue of hemoglobin.

Copper in erythrocytes appears to exist in two forms - one firmly bound to erythrocuprein, the other more loosely bound to the same protein.

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INTRODUCTION

The importance of metallic elements in biological systems has been recognized for many years. In some cases, such as iron in erythrocytes, the function of the metal is well established, and in other cases where the elemental function is less clear, such less desirable manifestations as disease due to metal deficiency may sometimes be eliminated by supplementation of the appropriate element in the diet. In many instances the metal concentration in the system is very low - perhaps well below one part per million - and these low levels have often hampered a study of the mechanism of action of a particular metal.

Since the essentiality of many metals at trace levels is well established it has often been assumed that they function in some enzymatic capacity. In fact the isolation of certain enzymes has often been followed by the discovery that the enzyme contains a metal as an integral part of its molecular structure. Such discoveries may be regarded as rather accidental and a more systematic means of studying the roles played by metal in biological systems would appear to be desirable. This must surely depend upon the ability to determine precisely the metal in question at trace levels. In recent years the development of very sensitive analytical techniques such as neutron activation analysis, atomic absorption spectroscopy and radioactive isotopic tracing has in many cases considerably simplified trace analysis.

In biological systems the trace metals are very often associated with some particular protein moiety, forming a macromolecule of precise molecular weight. In some cases the metal is bound directly to the protein chain, in others through a prosthetic group. In either case the metal may be regarded as firmly bound (metalloproteins) or loosely bound (metal-protein complexes). Regardless of type of binding the precise molecular weights of these proteins are perhaps their most important physical property. Consequently a suitable technique for the identification and isolation of metal-containing proteins might involve the fractionation of a mixture of proteins in order of molecular weight, and the subsequent determination of the metal in question in each fraction.

Molecular weight fractionation of protein solutions has traditionally been carried out by ultracentrifugation. However, this method is slow, rather expensive in terms of capital investment, and really only applicable on a micro or semi-micro scale. A relatively new technique - gel chromatography - has none of these disadvantages and provides fractionation of a protein mixture under very gentle conditions. This latter property is a real asset in the isolation of proteins since the classical separation methods involving systematic precipitation can easily lead to partial or complete denaturation of the desired protein.

The studies described in this thesis involve the fractionation of blood sera and hemolysates by gel chromatography followed by analysis of the fractions for copper and manganese by neutron activation analysis and the use of radioisotopic tracers.

Gel Chromatography

This technique ideally involves the fractionation of macromolecules on a column of a neutral granulated gel of strictly controlled pore sizes. If a mixture of macromolecules is applied to the column only those molecules small enough to enter the gel grains can do so. The larger molecules are restricted to the solvent surrounding the gel particles, and when the column is washed with solvent these molecules move faster down the column than the smaller ones which are retarded by diffusion into the gel particles. Consequently larger molecules have a shorter effective path length down the column, due to their exclusion from the stationary gel phase, and they are eluted ahead of the smaller molecules.

Although there were early reports of molecular size effects during ion exchange separations, and non-ionic materials have traditionally been used in adsorption analysis, the first uncharged gel was synthesised only in 1954 by Deuel¹. However, the most important discovery was that by Porath and Flodin² in 1959 of the properties of the cross-linked polymer of dextran and epichlorhydrin, later marketed as Sephadex. Careful control of reaction conditions led to control of the gel pore size upon swelling in aqueous solution, and thus polymers suitable for fractionation of various molecular size ranges became readily available. Most of the published literature on gel chromatography refers to cross-linked dextran gels, although polyacrylamide and granulated agar gels have also been used.

Several workers have developed empirical relationships between elution parameters on gel columns and the molecular size or

weight of the eluted substances. Some relationships were also arrived at by consideration of hypothetical structures for the gel matrix and these generally showed that, as would be expected, molecular elution volumes correlated with molecular size rather than molecular weight. Experiments with asymmetric proteins have confirmed that molecular shape has a definite effect on elution volumes, and it is now generally accepted that the Stokes radius of a molecule is the parameter which best correlated with elution volume. Consequently gel chromatography cannot accurately be used to directly estimate the molecular weight of a protein by calibrating a column with proteins of known molecular weight, although for molecules of similar shapes and densities reasonably good values for molecular weights have been obtained.

Perhaps the best theoretical description of the gel chromatographic process has been proposed by Ackers³ who demonstrated a linear relationship between the molecular radius and the inverse error function complement of the column partition coefficient, without postulating any particular geometrical structure for the gel matrix. Using this relationship:

$$a = a_0 + b_0 \operatorname{erfc}^{-1} \sigma \quad (1)$$

where a = molecular radius

a_0, b_0 = constants for a particular column

$$\sigma = \frac{V_e - V_0}{V_t - V_g - V_0}$$

where V_e = elution vol. for solute

V_0 = void vol. of column

V_t = total vol. of gel bed

V_g = vol. not accessible to solvent

erfc = error function complement

a column may be calibrated using only two proteins of known Stokes radius, and the size of an unknown particle can then be determined from its elution volume.

When the Stokes radius of a molecule has been obtained the molecular weight can either be estimated by assuming values for the partial specific volume and the frictional ratio of the molecule, or it can be determined accurately by combining the gel chromatographic data with a value for the sedimentation coefficient of the molecule, thus also obtaining an accurate value for the frictional ratio.

A recent paper⁴ has proposed the gel chromatography of protein fragments obtained by denaturation, as a molecular weight determination technique, since the denatured fragments are homologous in shape and consequently their elution volume on a given column is directly proportional to their molecular weight. However, this method seems to demand a prior knowledge of the number of such denatured fragments produced, and also requires that these fragments should be eluted in positions well apart from one another.

For the purposes of this present work, however, the most important property of gel chromatography is its outstanding ability to fractionate proteins in solution in order of size, over a wide size range. Consequently protein-bound metals in a biological fluid may be readily separated from ionic or peptide-bound metals without involving the use of any harsh chemical or physical techniques which might lead to denaturation of the protein. An excellent monograph by Determann⁵ covering the whole area of gel chromatography, particularly from the practical aspect, has recently been published.

Nuclear Analytical Techniques

Although the first paper describing the technique of neutron activation analysis appeared in 1936⁶, there was little development of the method until intense neutron fluxes, such as those generated in a nuclear reactor, became more readily available. The last ten years has seen an amazing growth in the use of neutron activation analysis, as its potential, particularly for trace analysis became apparent.

The general principle of the method involves the rendering radioactive of elements in the sample under analysis by bombardment with neutrons. The induced radioactive species may then be identified and quantitated either with or without chemical separation. The theory and practice of neutron activation analysis has been adequately covered in a number of books and review articles^{7,8}, and will not be further described here.

Copper and manganese were the elements under study in this work and their nuclear properties should be considered.

Copper exists in nature as two stable isotopes ^{63}Cu (69.1% abundant) and ^{65}Cu (30.9%). Each of these may be activated by thermal neutron irradiation to produce ^{64}Cu and ^{66}Cu by the reactions $^{63}\text{Cu}(n,\gamma)^{64}\text{Cu}$ and $^{65}\text{Cu}(n,\gamma)^{66}\text{Cu}$. The half-lives of these isotopes are 12.8 hr (^{64}Cu) and 5.1 min (^{66}Cu). The longer-lived ^{64}Cu is generally of more importance in radiochemistry than ^{66}Cu since its longer half-life, combined with higher thermal neutron capture cross-section ($4.5 \times 10^{-24} \text{ cm}^2$ vs. $1.8 \times 10^{-24} \text{ cm}^2$) and higher natural abundance render it more sensitive for activation analysis purposes,

and permits work to proceed at a more leisurely pace than is the case with the 5.1 min isotope.

^{64}Cu decays by electron capture (42%), beta (39%) and positron emission (19%) and this last decay mode is usually used for counting purposes since the positron-electron annihilation reaction produces two 0.511 MeV γ -rays at 180° to each other. These can be readily quantitated by γ -ray spectrometry.

Manganese is one of the elements for which neutron activation analysis is most sensitive and suitable. The stable element is monoisotopic, occurring as ^{55}Mn which has a large thermal neutron capture cross-section ($13 \times 10^{-24} \text{ cm}^2$). The product isotope, ^{56}Mn has a convenient 2.6 hour half-life, and decays by β -emission with the production of a 0.845 MeV γ -ray for every disintegration. This, too, may be counted by γ -ray spectrometry.

Radioisotopic tracing studies involving copper are limited to experiments of relatively short duration due to the short half-lives of the readily available radioisotopes of copper. The only longer-lived radio isotope than ^{64}Cu is the very expensive ^{67}Cu ($t_{1/2} = 58.5 \text{ hr}$) produced either by fission of arsenic or bismuth, or by the nuclear reactions $^{64}\text{Ni}(\alpha, p)^{67}\text{Cu}$ or $^{67}\text{Zn}(n, p)^{67}\text{Cu}$. Consequently copper tracer experiments are generally performed with ^{64}Cu which can be prepared either by the (n, γ) reaction on ^{63}Cu or by (n, p) on ^{64}Zn . The latter method permits the production of large quantities of carrier-free ^{64}Cu .⁹

Manganese tracing experiments usually involve the use of ^{54}Mn , which decays by electron capture, producing a γ -ray of energy

0.840 MeV, and has a half-life of 291 days. Since there is no stable ^{53}Mn isotope, ^{54}Mn is usually prepared by the reaction $^{54}\text{Fe}(n,p)^{54}\text{Mn}$.

Since the (n,p) reactions on zinc and iron are excellent methods for the preparation of carrier-free ^{64}Cu and ^{54}Mn respectively, they obviously also could lead to high copper and manganese values in neutron activation analysis of samples containing large quantities of zinc or iron. This effect might be particularly noticeable in the determination of manganese in erythrocytes, although generally the cross-section for (n,p) reactions in a thermal neutron spectrum is of the order of millibarns, rather than barns, as for (n, γ) reactions.

Trace Elements in Blood

Many workers have analysed a wide variety of biological materials for inorganic trace elements, and in fact some of the most intensively studied tissues such as blood have been analysed for almost every element in the periodic table. An extensive compilation of the data arising from such studies on whole blood, plasma and erythrocytes has been prepared by Bowen¹⁰.

Rather fewer publications describe a more detailed analysis of the distribution of trace elements with blood components. A number of papers measuring non-dialysable metal levels in plasma or serum have appeared¹¹⁻¹³, and there have been a few studies of specific protein-metal interactions^{14,15}. Such gentle protein separation techniques as electrophoresis¹⁶, gel immunochemistry¹⁷ and absorption on ion exchange gels¹⁸ have been used in research of this nature. However, the number of investigations of the most difficult and perhaps most important problem - the

systematic determination of the number and type of proteins containing trace elements in blood or any other tissue - may be counted on one hand. 18,52

Many studies on trace elements in blood have involved the use of radioisotopic tracers. Although these methods might produce valuable results, such studies in vitro must be regarded as of doubtful significance unless it can be shown that the in vitro situation is a true parallel to that extant in vivo. On the other hand, in vivo experiments involving radioisotopes have been criticised on the grounds that such studies usually involve loading the system with an atypical quantity of the element under consideration, possibly thereby inducing a change in that element's metabolism. Even the use of carrier-free materials may not completely overcome this argument. By the radioactive decay law

$$\frac{-dN}{dt} = \lambda N \quad \text{where} \quad \frac{-dN}{dt} = \text{rate of decay}$$

$$\lambda = \text{decay constant}$$

$$= \ln 2 / \text{half-life}$$

$$N = \text{number of atoms}$$

in sample

For a long-lived isotope such as ^{54}Mn or ^{65}Zn the quantity represented by 1 mc of truly carrier-free isotope may approach or exceed the normal levels in the blood. As an example consider 1 mc ^{54}Mn ($t_{1/2} = 291$ days)

$$\lambda = \frac{0.693}{291 \times 24 \times 60 \times 60} = 2.77 \times 10^{-8} \text{ sec}^{-1}$$

$$\text{and } N = \frac{-dN}{dt} \cdot \frac{1}{\lambda} = \frac{3.7 \times 10^7}{2.77 \times 10^{-8}} = 1.33 \times 10^{15} \text{ atoms}$$

or the weight of $^{54}\text{Mn} = \frac{1.33 \times 10^{15}}{6 \times 10^{23}} \times 54 = 1.2 \times 10^{-7} \text{ gm.}$

Since the latest literature figures for the manganese content of human plasma are $\sim 0.5 \text{ ngm/ml}^{19,20}$, if an adult with a blood volume of $\sim 5 \text{ l}$, 50% of which is plasma, is dosed with 1 mc carrier-free ^{54}Mn , the plasma manganese level is raised by 10%. If this same dose was administered to a rabbit with a typical blood volume of 300 ml, the plasma manganese content would be 150% above the normal level.

Even in cases where disturbance of the normal level is less severe the major difficulty lies in the obtaining of truly carrier-free isotopes.

The above objections to some previous methods of studying trace metals in biological tissue are not valid if the tissue under investigation is removed without contamination from the in vivo situation and subjected to the desired separation technique, which should involve the least possible disruption of the system. Following such separation the resultant fractions are analysed for the desired elements.

While this method sounds simple in theory the application of it is rather more complicated, and so far the literature contains only one report of a systematic investigation into blood metalloproteins¹⁸. In this study Himmelhoch et al. fractionated serum by gradient chromatography on DEAE-cellulose, and the fractions were analysed for metals by spark emission and atomic absorption spectroscopy. Iron, zinc, manganese and nickel were found reproducibly in specific fractions, and calcium, magnesium and strontium were also studied.

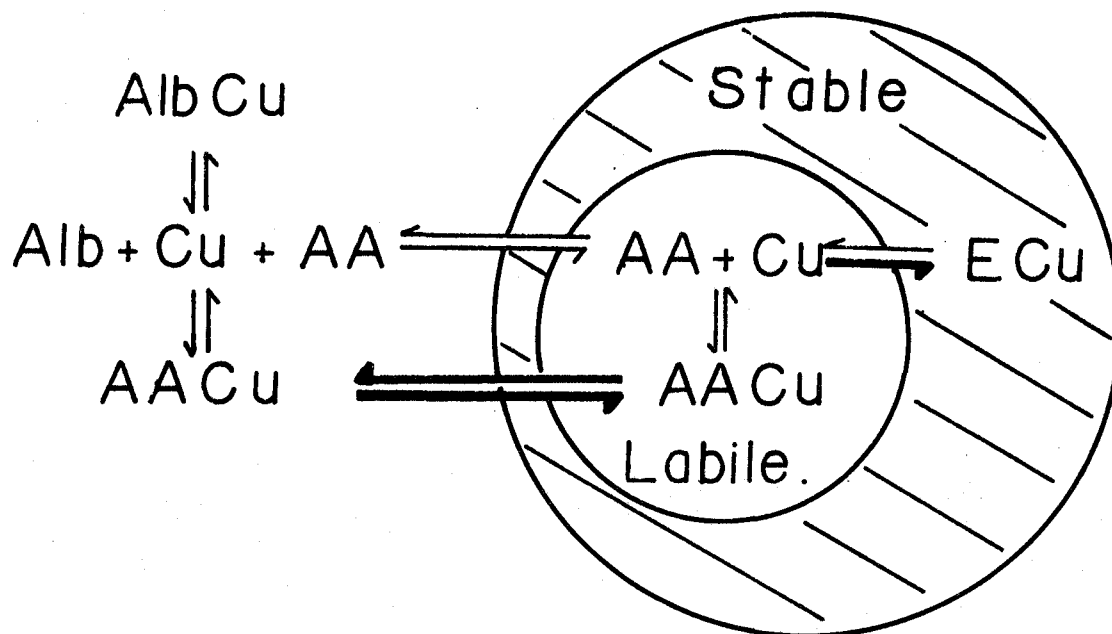
Copper in Blood

The normal copper concentration in mammalian blood has been well established at approximately 1 $\mu\text{gm/ml}$, about 50% being in plasma and 50% in erythrocytes. The copper distribution in plasma has been extensively studied and it has been shown that >90% of the metal occurs in the glycoprotein ceruloplasmin. This protein has been crystallised and its physical and biological properties are well characterised. Ceruloplasmin has a molecular weight of 151,000, contains eight copper atoms per molecule and has an axial ratio of 11. Copper in ceruloplasmin is not complexed directly by EDTA or diethyldithiocarbamate, and hence must be regarded as firmly bound to the protein structure. However, under suitable reducing conditions in vitro exchange of ceruloplasmin copper with radioactive copper has been demonstrated. A review of the metabolism of copper has been published by Sass-Kortsak²¹.

The small quantity of non-ceruloplasmin copper in serum or plasma is mainly complexed by albumin although a trace is also believed to be complexed by free amino acids. It has been suggested by Neumann and Sass-Kortsak²² that the albumin-bound copper, amino acid-bound copper and ionic copper are in equilibrium, and this system provides the basis of copper transport within the bloodstream. Following oral administration of ⁶⁴Cu, radioactivity appears in the serum, and reaches a maximum after two hours. At this stage almost all of the activity is bound to albumin. After 24 hours the albumin-bound activity has disappeared, and the ceruloplasmin blood fraction contains radioactive copper. Experimental evidence indicates that copper is incorporated into ceruloplasmin only during the protein's synthesis in liver microsomes, and based upon the above evidence the transport

mechanism proposed for the albumin-amino acid-ionic copper equilibrium seems reasonable.

The distribution of copper within the erythrocytes has been less intensively studied, and the situation in this case appears rather more complex. Mann and Keilin²³ isolated an intense blue copper protein from bovine erythrocytes in 1938, and a protein of similar molecular weight ($\sim 33,000$) and copper content (2 atoms Cu/molecule) was found in human erythrocytes in 1959, by Markowitz *et al.*²⁴ These workers stated that this protein, named erythrocuprein, accounted for virtually all of the erythrocyte copper, but later reported²⁵ that erythrocuprein contained only $\sim 60\%$ of the cellular copper. Still later Stansell and Deutsch²⁶ found that only 30-50% of the total erythrocyte copper was protein-bound (as erythrocuprein) and claimed that the remainder was dialysable. Further investigations by Neumann and Silverberg²⁷ showed that in vivo radioactive copper administered intravenously is very rapidly incorporated into erythrocytes reaching a peak less than 10 minutes after administration. This rapid diffusion is enhanced by the addition of free amino acids. Subsequently the erythrocyte activity declines then starts to increase again after about 24 hours. These results are interpreted by Neumann and Silverberg to indicate an active transport mechanism for ionic copper into erythrocytes and he implicates the amino acid-copper complex (see above) in this process by the following scheme:

PlasmaErythrocyte

The slow rise in erythrocyte ^{64}Cu activity after approximately 24 hours is related to the incorporation of the ionic erythrocyte copper into the erythrocyte copper in the scheme above. Hence the conclusions reached by Neumann and Silverberg are that erythrocyte copper occurs in two pools:

(i) a dialysable or labile compartment, whose copper arises from the albumin-bound copper in plasma, and which is made more freely diffusible through cell membranes by amino acids.

(ii) a non-dialysable pool which binds copper strongly with slow, primarily unidirectional copper turnover. This is the erythrocyte copper pool.

Manganese in Blood

The in vivo status of manganese in blood is considerably less

well understood than the copper situation, largely because of analytical difficulties arising from the extremely low concentrations of the element. Normal human whole blood manganese content is quoted in the recent literature as 8ngm/ml^{19,28}, but serum manganese levels are very much lower at ~ 0.5 ngm/ml^{19,20}. Over the past twenty years the reported serum manganese values have fallen steadily as more sensitive analytical techniques have been developed and the problems of contamination have been recognised, but not necessarily completely overcome.

In addition to analytical problems arising from the low serum manganese concentration, a further complication arises if radio-isotopic tracers are used in vivo to study manganese-binding proteins. Injected ionic manganese is cleared from the blood stream extremely quickly, most disappearing into the kidney and liver at a simple exponential rate with a half-life between 1.5 and 5 minutes³⁶. Consequently in vivo studies require large doses of radioisotope, even if in a carrier-free form, with the attendant dangers inherent in alteration of the normal manganese balance.

As stated previously, Himmelhoch et al.¹⁸ found a single manganese protein in serum during DEAE cellulose separation, but these results must be suspect due to the high manganese levels quoted. Based upon the figures quoted in the paper the measured protein-bound manganese content of serum was 40 ngm/ml, almost a factor of 100 above other literature values. Hence it is probable that these workers were observing the binding of manganese arising from contamination during the separation.

Cotzias has made an extensive study of manganese metabolism

and many of the measurements for manganese in blood, sera, etc. come from his laboratory. In 1960 Cotzias and Bertinchamps²⁹ reported the existence of a manganese binding β_1 -globulin which they named transmanganin in analogy to transferrin. A later publication from the same group³⁰ showed by ultrafiltration, dialysis, and ultracentrifugation experiments that virtually all serum manganese is protein bound. When serum dosed with carrier-free ^{54}Mn was subjected to electrophoresis the protein-bound radioactivity was overwhelmingly associated with the β -globulin fraction. When this fraction was split further electrophoretically into β_1 and β_2 the protein-bound activity migrated almost exclusively with the β_1 group of globulins. Similar results were obtained by electrophoresis of serum from rats which had been injected with ^{54}Mn prior to exsanguination. In all these experiments only a trace of radioactivity was associated with the albumin fraction. As a result of these studies it was concluded that it was a reasonable hypothesis that there was only one manganese-binding protein in serum, and that this was a β_1 -globulin. This was not identified in this publication as transmanganin. However, the results of Himmelhoch *et al.* (see earlier) were cited in support of this hypothesis.

Mokeysovska³¹ has shown that manganous ions form a labile complex with human serum albumin, which decomposes to its original components during Sephadex gel chromatography. By saturating the column with albumin she was also able to show that the elution volume of manganese shifted from that of free manganese ions to that of the protein, as determined previously.

In other studies Fritze and Robertson²⁰ showed by desalting on a gel filtration column, and subsequent neutron activation analysis, that virtually all human serum manganese was protein bound, thus confirming the similar finding by Cotzias.

Fritze and Gietz³² added various metals in the form of radioisotopes to pig serum in vitro then fractionated samples on gel chromatographic columns. They showed that copper, zinc, iron, chromium and gallium were complexed by various serum proteins, but found no evidence for pick-up of manganese. However, this metal was not used carrier-free, and in fact 50 µgm Mn(II) was added to 0.4 ml serum. The total activity of the sample was ~ 280,000 cpm, and these results considered together with those of Cotzias¹ merely indicate that the binding capacity of the manganese-binding protein in serum is very low, i.e., binding of 1 ngm Mn/ml serum would have given a ⁵⁴Mn activity in the protein region of Fritze and Gietz's separation ~ 30 cpm, whereas the ionic activity would have been ~ 280,000 cpm.

Although erythrocytic manganese levels are 10-20 times higher than those in plasma, analysis by neutron activation methods are hampered by interference from the nuclear reaction $^{56}\text{Fe}(n,p)^{56}\text{Mn}$ arising from the large quantities of heme iron present (~1.1 mgm/ml red cells). Some workers have investigated this interference and have found that the n,p reaction makes a 30-40% contribution to the total ⁵⁶Mn produced during irradiation^{28,33}. This naturally depends upon the degree of thermalisation of the reactor neutrons, but the claim by Cotzias et al.¹⁹ that in their determination of manganese in

whole blood by activation analysis the contribution to ^{56}Mn from ^{56}Fe could be neglected must be suspect, particularly in view of the data given in the paper.

The interest in erythrocytic manganese arises from the important hypothesis by Borg and Cotzias³⁴ that a naturally occurring manganese porphyrin may exist in red cells. If this were so it would be the first time such a manganese chelate had been found in nature, and might implicate manganese in hemoglobin formation, particularly since one of the symptoms of manganese deficiency in rats is breakdown of hemoglobin regeneration.

Borg and Cotzias found that after intravenous injection of ^{54}Mn in humans and laboratory animals the rate of appearance of the isotope in the erythrocytes was similar to that observed for ^{59}Fe , and even more closely related to the rate of appearance of protoporphyrin IX labelled with ^{15}N . Furthermore, once incorporated ^{54}Mn could not be removed by dialysis or by treatment with EDTA, but most could be recovered in a crystalline hemin preparation. These results were later verified by other workers^{35,36} but no isolation of this manganese compound has yet been reported. Consequently the evidence for the existence of this manganese porphyrin remains somewhat circumstantial.

The reported rate of incorporation of ^{54}Mn appears to be approximately the same as the rate of production of red cells, so the manganese could well be present as some other macromolecule than a porphyrin. The inclusion of ^{65}Zn in erythrocytes follows a somewhat similar pattern but the zinc is known to be associated with carbonic anhydrase³⁷. In many cases where metals are bound to protein they become an integral part of the molecular structure and are non-

dialysable and unaffected by their normal complexing agents - examples such as copper in ceruloplasmin and iron in transferrin may be cited. Hence the most conclusive evidence for the existence of the manganese porphyrin lies in the ^{54}Mn occurring in crystalline hemin preparations, even though a recent study³⁶ showed a recovery of only 60-70% of erythrocyte ^{54}Mn with hemin.

EXPERIMENTAL EQUIPMENT AND PROCEDURES

(a) Materials and Reagents

All gel chromatography in aqueous solution was carried out on cross-linked polyacrylamide Bio-Gels (Bio-Rad Laboratories, Richmond, California). These materials are available in a similar series of molecular weight fractionation ranges, and mesh sizes as the more generally used dextran gels, and were chosen ahead of the latter on the basis of semi-quantitative activation analysis experiments which showed the polyacrylamide materials to contain less copper and manganese than did the dextran gels.

The designation P-X indicates that the gel has an exclusion limit such that globular proteins of molecular weight X,000 are eluted with an R_f of 0.9.

Two batches of Bio-Gel P-150 were used in these studies and each produced a characteristic serum protein chromatogram. The first material (#37473, 14/6/66) gave rise to protein fractionations as in Figs. I-III, while the second batch (#44613, 13/1/67) produced chromatograms as in Fig. IV. The flow rates of columns packed from batch #44613 were significantly greater than those prepared from the older material, whose properties appeared to be more those of Bio-Gel P-200, than P-150, as these are described in the Bio-Rad Laboratories catalogue.

Blue dextran and ^{24}Na solutions were used to measure the void volume and total column volume available to the solute respectively. For non-aqueous gel chromatography Bio-Beads SX-8 (Bio-Rad Laboratories) was used.

Analytical grade reagents were used without further purification throughout this work, with the exception of the chromatographic eluting solution (0.15 M ammonium acetate solution, pH \sim 7) which was prepared by dilution of Aristar grade ammonia solution and Aristar grade acetic acid (B.D.H. Ltd., Toronto, Ont.) with deionised distilled water. This high purity ammonium acetate solution was stored in polyethylene.

Irradiation capsules for neutron activation analysis experiments were either quartz or polyethylene, depending upon the planned irradiation time. Quartz ware was washed in hot concentrated nitric acid for 24 hours prior to use, and rinsed thoroughly with deionised water. Polyethylene capsules were rinsed in deionised water alone.

Radioisotopic tracers were generally prepared in the McMaster Reactor (thermal flux $\sim 2 \times 10^{13}$ n/cm²/sec), although one sample of carrier-free ^{54}Mn was purchased from New England Nuclear Corp. (Boston, Mass.). A further sample of carrier-free ^{54}Mn was prepared by irradiation of spec-pure iron in the McMaster Reactor for approximately six months, followed by four months cooling. The sample was leached in acid then dissolved in 6 M redistilled hydrochloric acid solution. The iron was oxidised to Fe(III) by prolonged bubbling of Cl_2 gas over the surface of the boiling solution, and then separated from manganese by absorption onto a Bio-Rad Analytical Grade anion exchange resin AG 1 x 10. The Mn(II) was eluted with 6 M HCl soln. and

collected in a perchloric acid-washed teflon beaker where it was evaporated to dryness in a dust-free atmosphere. After repeating the procedure, evaporating to dryness, and fuming the residue with conc. perchloric acid virtually no residue was obtained. The yield of ^{54}Mn was approximately 1 mc, and the radiochemical purity was >99.99%. After the few months cooling period there was no detectable ^{51}Cr produced by the reaction $^{54}\text{Fe}(n,\alpha)^{51}\text{Cr}$. Whether such a sample of ^{54}Mn was truly carrier-free could be determined only by mass spectrometry.

(b) Instrumentation

During gel chromatography of protein solutions the eluate protein content was monitored with a LKB Uvicord absorptiometer ($\lambda = 253 \text{ m}\mu$), and eluate fractions were collected on a LKB Ultra-Rac fraction collector.

Irradiations for neutron activation analyses were carried out in the McMaster Reactor (thermal flux $\sim 2 \times 10^{13} \text{ n/cm}^2/\text{sec}$, cadmium ratio ~ 20). Some short-term irradiations were done using the pneumatic rabbit system, at a slightly reduced flux.

Flux variations were monitored by the use of standards. In some experiments cobalt as a 1% alloy in aluminum wire was used merely as a flux monitor.

In other experiments the standards for copper or manganese determinations were used. For copper analyses Alcan alloy 424 (3.7% Cu) obtained from Aluminum Company of Canada was used.

Household aluminum foil containing 70 ppm manganese was used as a standard for manganese determinations.

All radioactivity measurements were carried out by gamma ray

spectroscopy, using one of the following:

(i) a 7.5 x 7.5 cm NaI(Tl) crystal in conjunction with a Victoreen DD2 amplifier and a Nuclear Data 256 channel analyser.

(ii) a co-axial Ge(Li) detector with an active volume of 12 cm³, in conjunction with an Ortec FET preamplifier, Ortec amplifier and Nuclear Data Series 2200 1024 channel analyser. This system had an energy resolution of 5.4 keV at the 1.118 MeV ⁶⁵Zn line, with a photopeak to Compton scatter ratio of 6.

(iii) a 5 cm diameter NaI(Tl) crystal with a 1.8 cm diam. well in conjunction with a Tracerlab single channel analyser and Hamner scaler.

(c) Column Preparation

All gel chromatographic columns were prepared in the generally-accepted manner, i.e., the dry material was swollen in the eluting buffer for 24-48 hours, high exclusion limit gels were deaerated under vacuum, the gel was added to a silicone-coated column as a slurry, allowed to settle by gravity, and then washed with several column volumes of eluting buffer to stabilise the gel bed. However, since these columns were to be used in trace metal analysis these steps were sometimes modified to some extent. In columns designed for copper experiments the 0.15 M ammonium acetate swelling solution was made 0.01 M with respect to CN⁻ and the pH readjusted to ~ 7 with acetic acid. When columns were to be used for manganese analysis hydroxylamine hydrochloride was added to make the swelling solution ~ 0.05 M in that reagent, and the swollen gel was boiled gently in

this solution for ~ 1 hr. The first precaution was to complex any copper present in the dry gel, the second to dissolve any MnO_2 which seemed the most probable cause of manganese contamination within the gel.

After packing, the columns were washed with 20-50 column volumes of high-purity 0.15 M ammonium acetate solution added to the column through a quartz reservoir through a bed of Chelex 100 (Bio-Rad Laboratories) in the case of copper experiments, and through a bed of AG 50W x 12 cation exchanger (Bio-Rad Laboratories) for manganese experiments. Columns prepared in such a manner were, hopefully, "ultra-clean".

(d) Sample Preparation

Human serum and erythrocytes, rabbit plasma, serum and erythrocytes and rat plasma were all used at various stages of this work. Human blood was used for all the activation analysis work for copper, rabbit blood for all the manganese determinations, and rat blood in the preliminary feasibility experiments. The animals were used wherever radioactive tracers were involved, and the choice of animal depended upon the number of samples required. The manganese studies required repeated blood sampling, hence the use of a rat was clearly impracticable. All blood samples, other than those in the feasibility studies, were obtained by cannulation of a vein or artery in order to minimise the possibility of sample contamination. For copper experiments a polyethylene cannula was inserted approximately 15 cm into the forearm vein of an adult male through an "Intracath" assembly. The needle was withdrawn leaving the cannula in place. Blood was allowed to drain through the cannula and the first 10 ml was rejected to allow adequate flushing of the system. Blood samples

were collected in quartz centrifuge tubes. Rabbits were cannulated either in the ear vein, if a small blood sample was needed, or in the carotid artery if a quantity greater than 5 ml was required.

Serum was prepared by allowing blood to coagulate at 37°C for 4 hours then centrifuging at a slow speed. Serum showing any evidence of hemolysis was discarded.

Plasma/erythrocyte preparations for copper determination was carried out using heparin as anti-coagulant. For manganese analyses acid citrate dextrose solution was used in place of heparin since our own semi-quantitative experiments confirmed published data showing a high manganese concentration in commercial heparin preparations²⁸.

Hemolysates were prepared by centrifuging the erythrocytes down from anti-coagulant treated blood, and pipetting off the plasma. The cells were washed three times in isotonic saline solution (stored over Chelex 100) and then hemolysed with an equal volume of deionised, distilled water. The mixture was agitated gently and then allowed to stand for at least 1 hr at 5°C. Stroma were removed by centrifugation.

(e) Gel Chromatography

Gel chromatographic fractionations were carried out by allowing the buffer above the gel bed to run through until the surface was dry. The serum or hemolysate sample was then applied to the surface through a quartz pipette, and allowed to soak in. The sample was then washed further into the bed with two small portions of the eluting buffer, before the reservoir was replaced on the top of the column, and elution allowed to proceed in the usual manner.

Eluate fractions for activation analysis were collected in either quartz or polyethylene capsules, and immediately frozen. In most cases the samples were subsequently lyophilized prior to irradiation, though some manganese analyses were performed by short irradiation of liquid samples.

(e) Post Irradiation Treatment

Most samples were irradiated in the dry state, and generally irradiation of liquid samples produced protein precipitation so in almost all cases it was necessary to leach the active material from the irradiation capsule. This was generally done with a hot concentrated nitric acid solution of the appropriate carrier. If chemical separation was intended prior to counting the volume of acid solution used was measured by pipette, whereas for samples counted without any chemical separation volumes were merely adjusted to a constant value after leaching.

Preliminary experiments showed that two leaches were sufficient to remove all (>99%) of the material dried inside a capsule. Experiments with empty capsules showed a small quantity of copper activity was leached from the quartz, and this together with copper in the eluent amounted to a blank of ~ 3 $\mu\text{g}/\text{fraction}$.

Polyethylene irradiation capsules gave a lower blank but suffered from the disadvantage that they became very brittle if irradiated for more than 30 minutes or so. However, these containers were used throughout for manganese analyses, and also for some copper determinations.

(f) Chemical Separations

If counting by NaI(Tl) was intended, separation of copper or manganese was necessary. The following schemes were used:

(a) Copper: The combined leach solutions were added to concentrated H_2SO_4 and the mixture was boiled down to the evolution of SO_3 fumes. If any carbon precipitated, fuming nitric acid was added to the boiling mixture until a clear solution was obtained. After cooling and neutralisation with conc. NH_4OH solution, NaCl was added as a sodium hold-back carrier. Copper, together with other transition metals was absorbed onto a 10-fold excess of Chelex-100 by stirring for 10 min. The absorbed metals were then washed off the chelating resin with 2 N H_2SO_4 solution, the pH was adjusted to ~ 2 and copper was extracted into chloroform as the cupferrate³⁸. This procedure gives a chemical yield 95% and separates copper from the other activities present in the sample - mainly ²⁴Na, ⁸²Br, ⁵⁶Mn and ⁶⁹Zn.

(b) Manganese: The combined leach solutions were boiled for 15 minutes. After cooling, saturated $KBrO_3$ solution was added and the solution was again boiled to precipitate MnO_2 which was centrifuged down. The supernatant was decanted, the precipitate dissolved in concentrated HNO_3 plus 1 drop 30% H_2O_2 solution, and more saturated $KBrO_3$ solution added. Upon boiling MnO_2 was again precipitated. After cooling the precipitate was filtered onto a weighed glass fibre filter, washed with H_2O , dried, weighed and counted. The chemical yield in this procedure was typically 80-90%.

RESULTS AND DISCUSSION

The experimental results reported here are in chronological order. Following the success of the radioisotopic feasibility studies described below, it was intended to apply the gel chromatographic fractionation and subsequent neutron activation analysis to the copper distribution in serum which has been intensively studied and would therefore serve as a model system. The method would then be applied to manganese in serum and erythrocytes. It was finally applied to copper in erythrocytes to give the overall blood distribution for both copper and manganese.

(1) Feasibility Studies

To test the feasibility of the protein fractionation scheme a rat was dosed with carrier-free ^{64}Cu and after twenty-four hours a blood sample was taken. According to the literature by this time the only copper activity in the serum should have been incorporated in ceruloplasmin. When a serum sample was fractionated on Bio-Gel P-150 the ^{64}Cu activity was related to the protein chromatogram as is shown in Fig. I. The three peaks in the protein distribution have been

In all figures the solid line represents the protein elution distribution, the broken line the metal concentration or radioisotope activity as appropriate. Such experimental details as column size, sample vol., etc. are given in Appendix II. In the interests of clarity, error bars have been omitted except where they are significant. In most of such cases only one is shown. All results shown have been corrected for isotope decay, and reactor flux variations where necessary.

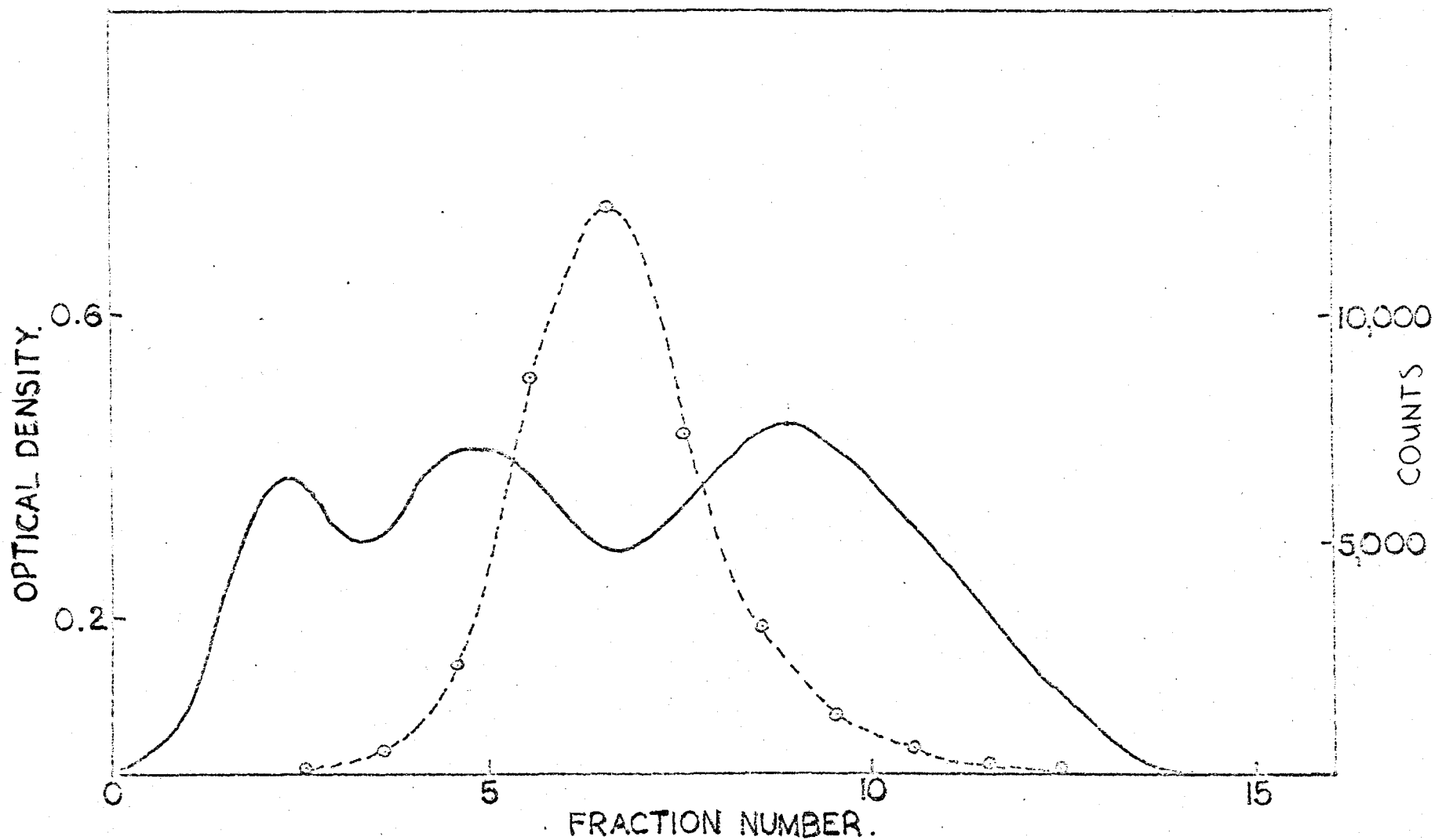


FIG I

0.4 ml rat serum applied to Bio-Gel P-150 (#37473) column 27 x 1.6 cm. Fraction size 1.5 ml. Rat dosed with ^{64}Cu 24 hours prior to blood sampling.

assigned by Porath³⁹ as follows: the first (left-hand) peak contains the high molecular weight serum components (α - and β -lipoproteins and α - and β -globulins), the centre peak γ -globulin, and the third peak albumin. The typical plasma elution chromatogram also contains three such peaks, though in this case the high molecular weight peak also contains fibrinogen.

It can be seen that only one copper activity peak was obtained and this appeared at an elution position corresponding to a lower molecular size than the γ -globulin peak. Based upon literature results this peak must represent the ceruloplasmin. Although ceruloplasmin and γ -globulin have similar molecular weights (150,000) the difference in elution volumes may be attributed to the assymetry of the ceruloplasmin molecule (axial ratio = 11⁵⁸ compared with the γ -globulin axial ratio of 5⁵⁹). Killander⁴⁰ in the course of in vitro experiments involving the addition of ceruloplasmin to serum observed the same relative positioning of the ceruloplasmin peak with respect to the γ -globulin and albumin peaks after gel chromatography on Sephadex G-200.

No sign of ⁶⁴Cu activity was found in the albumin region, indicating that all of the injected ⁶⁴Cu remaining in the serum had been incorporated into ceruloplasmin. The almost Gaussian-shaped activity elution curves were an important feature of these preliminary experiments. Such curves were obtained even when the protein chromatogram peaks were distorted by overloading of the column (Fig. II) and provided evidence that even if such overloading results in rather amorphous chromatograms the elution of an individual protein is essentially unaffected.

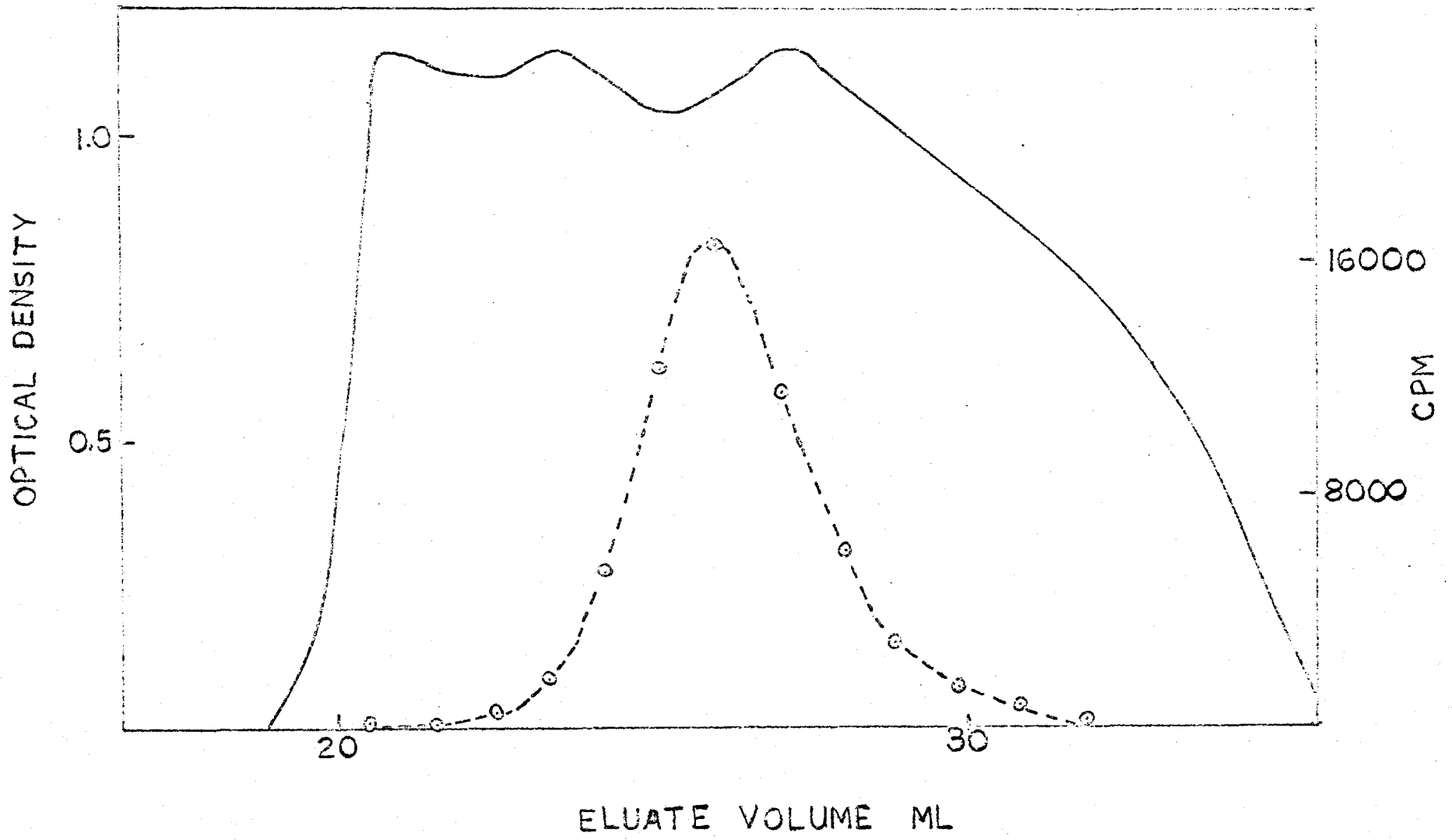


FIG II

Rat plasma labelled in vivo with ^{64}Cu on P-150 column (column overloaded with protein)

Further feasibility experiments involved the in vivo labelling of serum proteins with ^{131}I , and Fig. III shows the ^{131}I distribution compared with the protein distribution after fractionation of rat plasma on P-150. The blood sample was taken 48 hours after injection of the isotope and as can be seen, by this stage there were two regions containing ^{131}I in the protein chromatogram together with a large peak representing slightly more than 50% of the isotope in the ionic region.

It should be noted that the larger ^{131}I peak in the protein region does not line up with the albumin peak but appears at a slightly lower molecular size position. This is in accord with the recently published data of Miyai et al.⁶⁰ who reported that thyroxine, into which ^{131}I is incorporated is bound by three plasma proteins, the main one being pre-albumin which has a molecular weight of 61,000 (compared with 70,000 for albumin). The other thyroxine binding proteins are an α -globulin and albumin.

Activation Analysis for Copper in Serum

Fig. IV shows the protein elution curve (solid line) for a typical human serum fractionation on Bio-Gel P-150, together with the copper concentration (broken line) in each fraction as determined by neutron activation analysis. 2 ml serum were applied to a column 45 x 2.6 cm and 2.5 ml eluate fractions were collected. Over 90% of the copper is contained in a single peak positioned between the γ -globulin and albumin peaks. Based upon evidence from the ^{64}Cu tracer work this peak is attributed to the ceruloplasmin content of the serum. A shoulder was found on the lower molecular size side of the ceruloplasmin peak in all four of the activation analysis runs

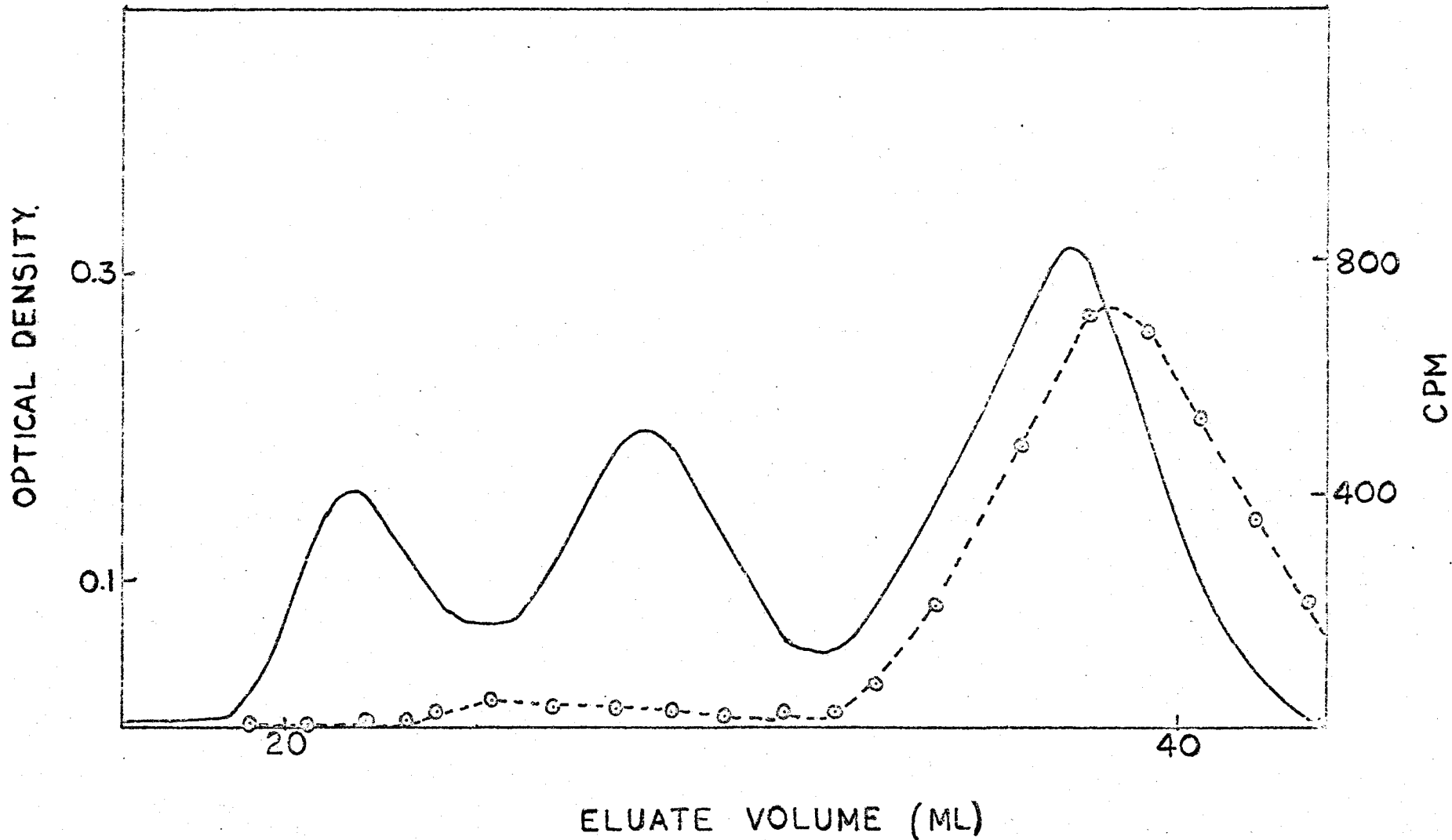


FIG III

0.5 ml rat plasma applied to Bio-Gel P-150 (#37473) column 31 x 1.6 cm. Fraction size ~1.5 ml. Rat dosed with ^{131}I 48 hours prior to blood sampling.

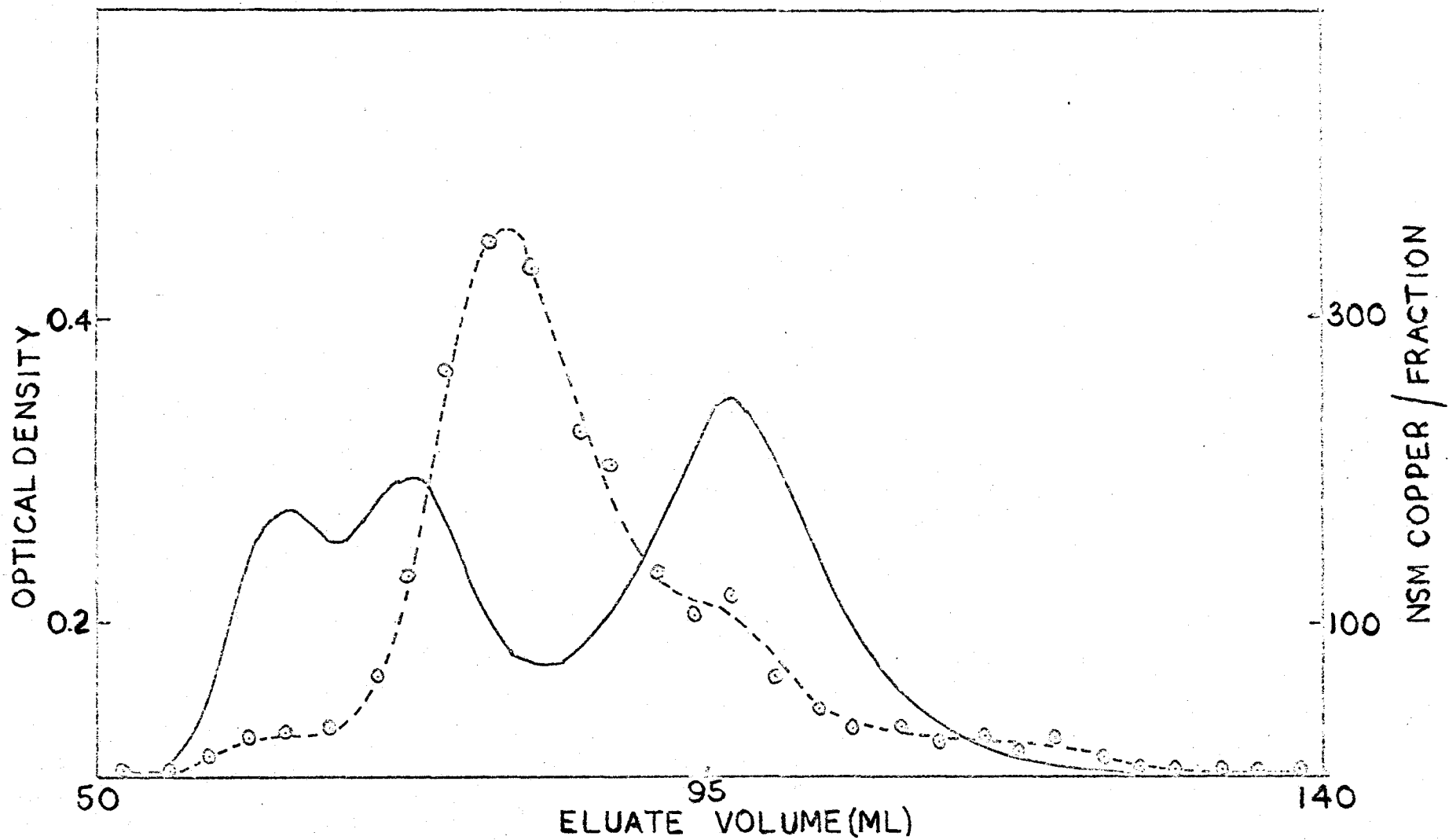


FIG IV

2 ml human serum applied to Bio-Gel P-150 (#44613) column 45 x 2.6 cm. Fraction size 2.5 ml. Copper in eluate determined by neutron activation analysis.

performed. This small quantity of copper (<10% of the total) occurs in the same position as the albumin in the protein chromatogram, and therefore presumably represents the albumin-bound copper.

A smaller peak is visible before the main copper peak and this is probably due to contamination picked up during the gel chromatographic separation. In vitro addition of ^{64}Cu to serum, and subsequent protein fractionation results in a protein and activity distribution as shown in Fig. V. Over 99% of the activity complexed by protein is found in the albumin region of the protein chromatogram, but a very small amount of ^{64}Cu is found at the high molecular size end of the protein elution. This particular in vitro type of experiment has been extended to other elements and the results reported elsewhere by Fritze and Gietz³². In an early activation analysis run (Fig. VI) a copper peak comparable in size to the ceruloplasmin peak was observed at the beginning of the protein elution. A mass balance for copper indicated that this peak was due to contamination since the total copper found was ~ 2 $\mu\text{gm/ml}$ or twice the accepted serum copper content. Since this first peak had the characteristic shape produced by gel chromatography of a single protein, and covered several fractions the eluate must have been contaminated prior to the fraction collection stage of the procedure. However, the contamination must also have occurred after application of the sample to the chromatographic column as there was virtually no enhancement of the albumin-bound copper peak. Consequently, the only explanation for this effect is the scavenging of traces of copper from the column by a high molecular weight protein in the serum. In subsequent work the cyanide washing

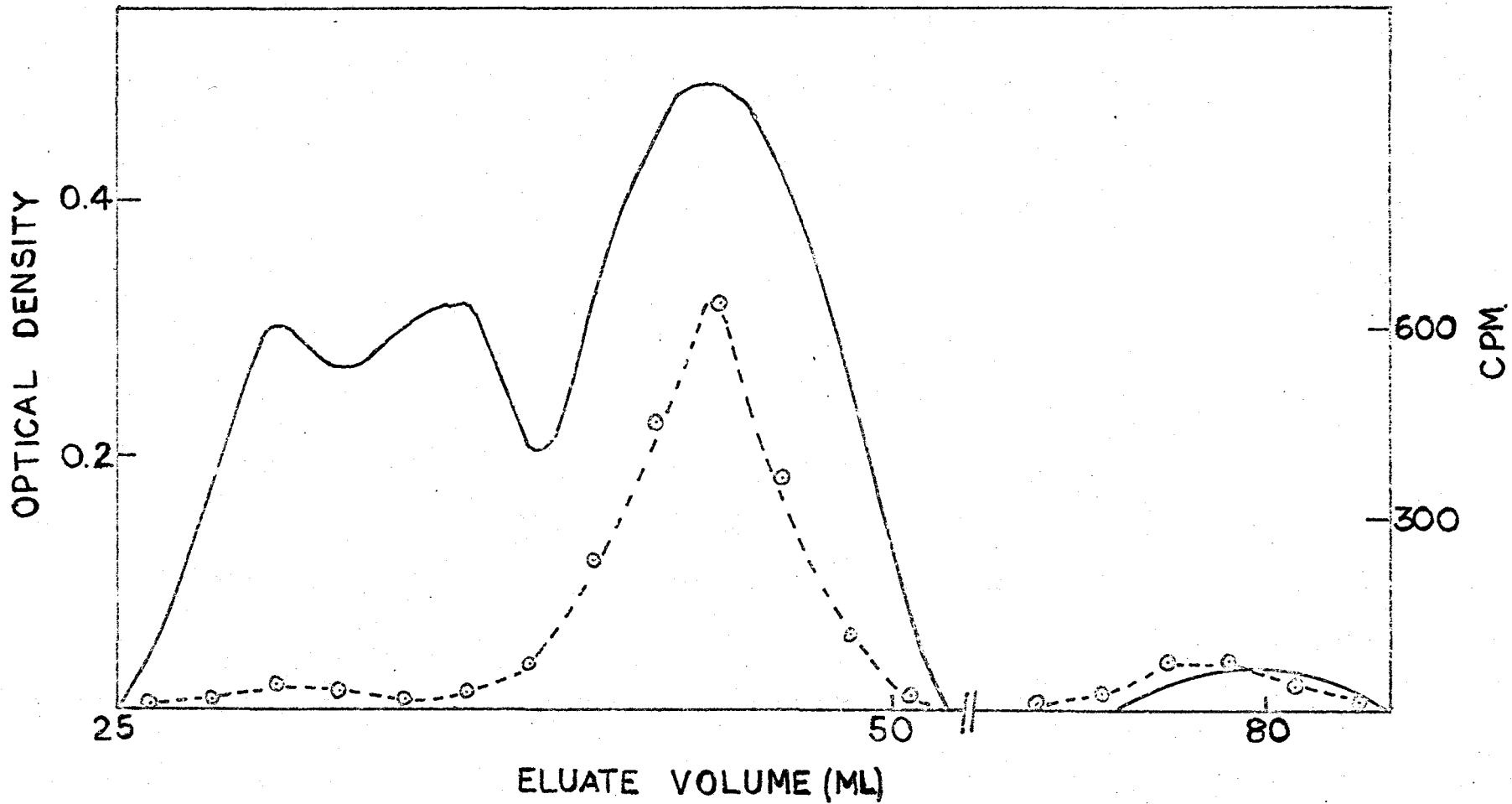


FIG V

2.5 ml human serum applied to Bio-Gel P-150 (#37473) column 44 x 2.6 cm. Fraction size 2.5 ml. Copper in eluate determined by neutron activation analysis and expressed as normalised counts ^{64}Cu per minute.

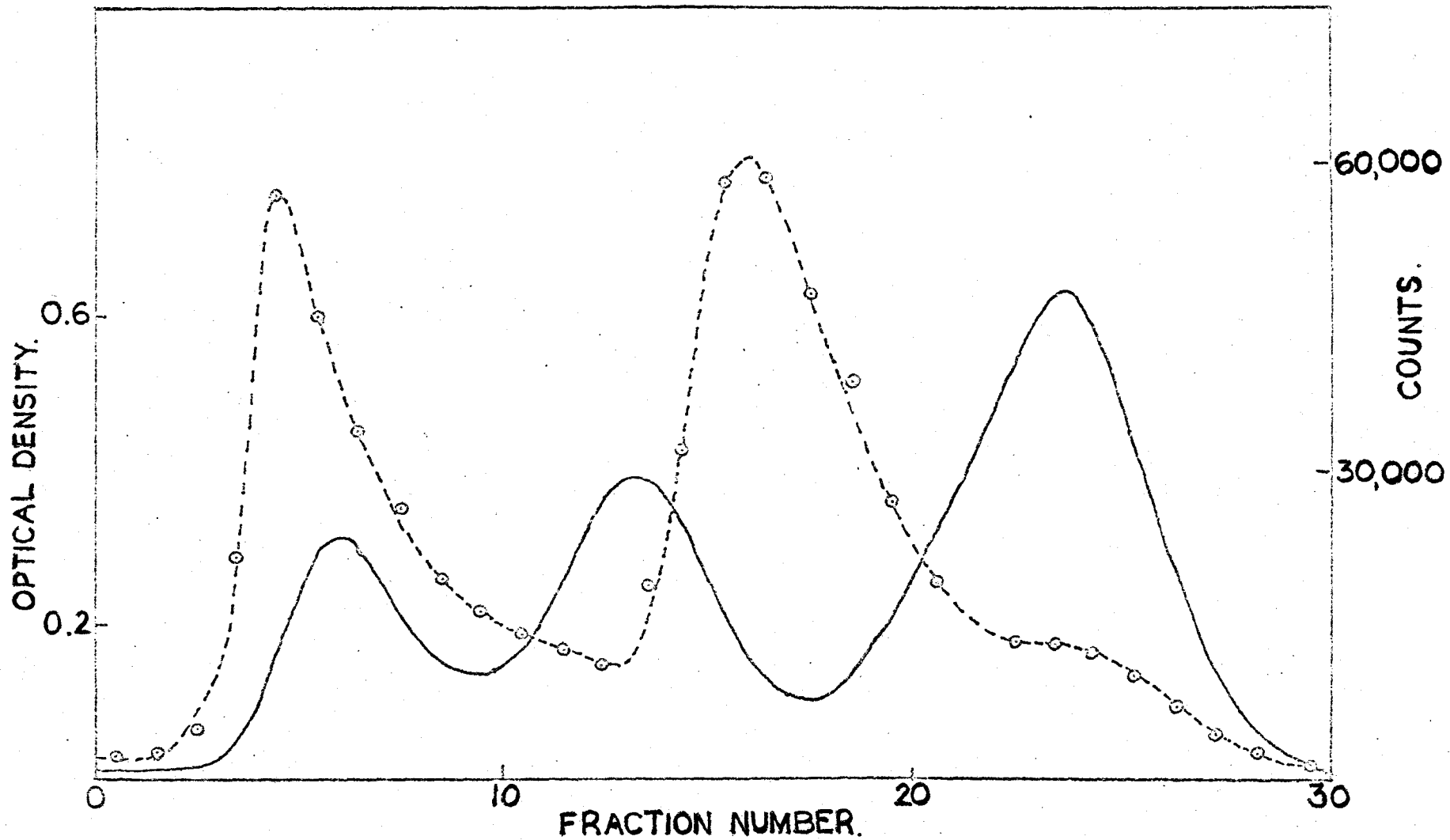


FIG VI

0.5 ml human serum + ^{64}Cu in 160 μg m Cu (II) solution applied to Bio-Gel P-150 (#37473) column 32 x 1.8 cm. Fraction size 2 ml. Serum + isotope incubated for 30 min. prior to application to column.

step was introduced during the column preparation (see experimental section) and this appeared to have the desired effect in almost eliminating this peak (see Fig. IV). Despite this argument the possibility that the first shoulder in Fig. IV represents a high molecular weight copper protein complex existing in vivo cannot be discounted.

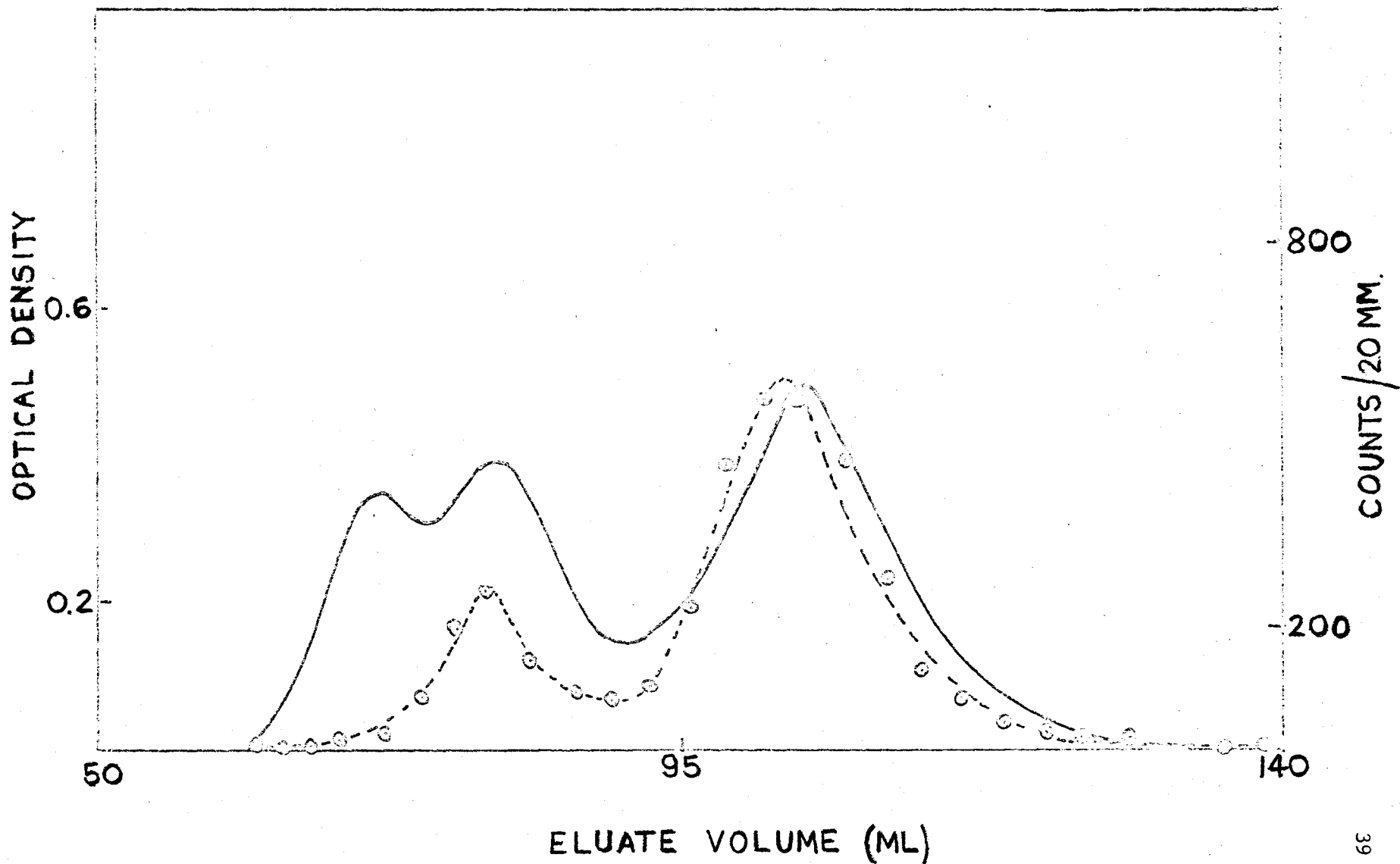
Apart from the sample "blanks" (experimental section) the possibility of the production of ^{64}Cu by any other reaction than $^{63}\text{Cu}(n,\gamma)^{64}\text{Cu}$ must be considered. The only likely other contributory process would be $^{64}\text{Zn}(n,p)^{64}\text{Cu}$, and although preliminary experiments similar to those reported here have shown some zinc bound in the albumin region of the protein chromatogram, the quantities found and the low cross-section for the (n,p) reaction make any such contribution to the ^{64}Cu activity negligible. From unpublished work⁴² using the McMaster reactor it has been shown that 1 μgm zinc "simulates" 1.5 ngm copper, because of the n,p reaction during neutron activation analysis. Since total zinc and copper levels in serum are approximately the same the n,p reaction could only cause significant interference if the bulk of the protein-bound zinc occurred in a region of the chromatogram where the copper level was very low. The total copper determined in the fractionation shown in Fig. IV represents a serum copper content of 1.1 $\mu\text{gm/ml}$, well within the accepted physiological limits.

Iron Distribution in Serum

In one protein fractionation/activation analysis run the samples were irradiated for seven days, in an attempt to measure copper, zinc and silver simultaneously. The zinc and silver results

will be reported by Hancock and Fritze⁴³ elsewhere. After the ⁶⁴Cu activity had been counted on a Ge(Li) detector without chemical separation the samples in nitric acid and containing 1 mg Fe carrier were evaporated to dryness. The residue was redissolved in 9 M H₂SO₄ solution and 1 mgm Ag carrier was added. The solutions were again taken to near dryness and then diluted to ~30 ml with water. These solutions, now ~0.1 M in H₂SO₄ were applied to Dowex AG 50W x 12 cation exchange resin columns (6 x 40 mm) in Pasteur pipettes. The iron and silver were absorbed on the column which was washed with 4 column volumes H₂O to remove ³²PO₄³⁻. The Fe(III) and Zn(II) were eluted from the columns with 2 M HCl solution and precipitated AgCl was subsequently washed off with CN⁻. The combined Fe/Zn solutions were counted on a NaI(Tl) detector. The iron distribution in relation to the protein chromatogram is shown in Fig. VII. Two iron peaks were detected and these were attributed to the two known serum iron proteins, transferrin and the haptoglobin-hemoglobin complex. Transferrin was originally reported to have a molecular weight of 90,000, but recent studies have amended this value to 70,000⁴⁴. On this basis the lower molecular size iron peak, which in fact almost coincides with the albumin peak in the protein chromatogram is assumed to represent the serum transferrin iron content.

The haptoglobins in serum combine with free hemoglobin to form stable complexes. Starch gel electrophoresis experiments⁴⁵ have shown that three types of haptoglobins, designated 1-1, 2-1 and 2-2, exist, and furthermore that the type of haptoglobin in an individual is characteristic and genetically determined. Type 1-1 binds one mole hemoglobin/mole haptoglobin and has a molecular weight ~85,000 -



ELUATE VOLUME (ML)
FIG VII

2 ml human serum applied to Bio-Gel P-150 (#44613) column 45 x 2.6 cm. Fraction size 2.5 ml. Iron in eluate determined by neutron activation analysis, expressed as normalised counts ^{59}Fe per 20 minutes.

hence the complex molecular weight is $\sim 150,000$, and this is presumably the origin of the iron peak of approximately that molecular weight in the gel chromatographic experiment. Types 2-1 and 2-2 complexes would have higher molecular weights ($>200,000$) and would be expected to appear in the first protein peak at the column exclusion limit.

Manganese in Serum

Following the success of the copper experiments it was decided to apply the same technique to an analysis of manganese in serum. Early experiments very quickly showed that the major obstacle in this study would be the contamination problem. To take a serum sample containing only ~ 0.5 ngm protein-bound manganese per ml and to further fractionate this would require exquisite control of manganese contamination.

Many runs were carried out but only a few produced satisfactory results and even then the drawing of worthwhile conclusions was difficult. Since it was necessary to use many blood samples, and the "Intracath" sampling method (experimental section) as applied to human subjects involved considerable stress, it was decided to use rabbit blood in this study.

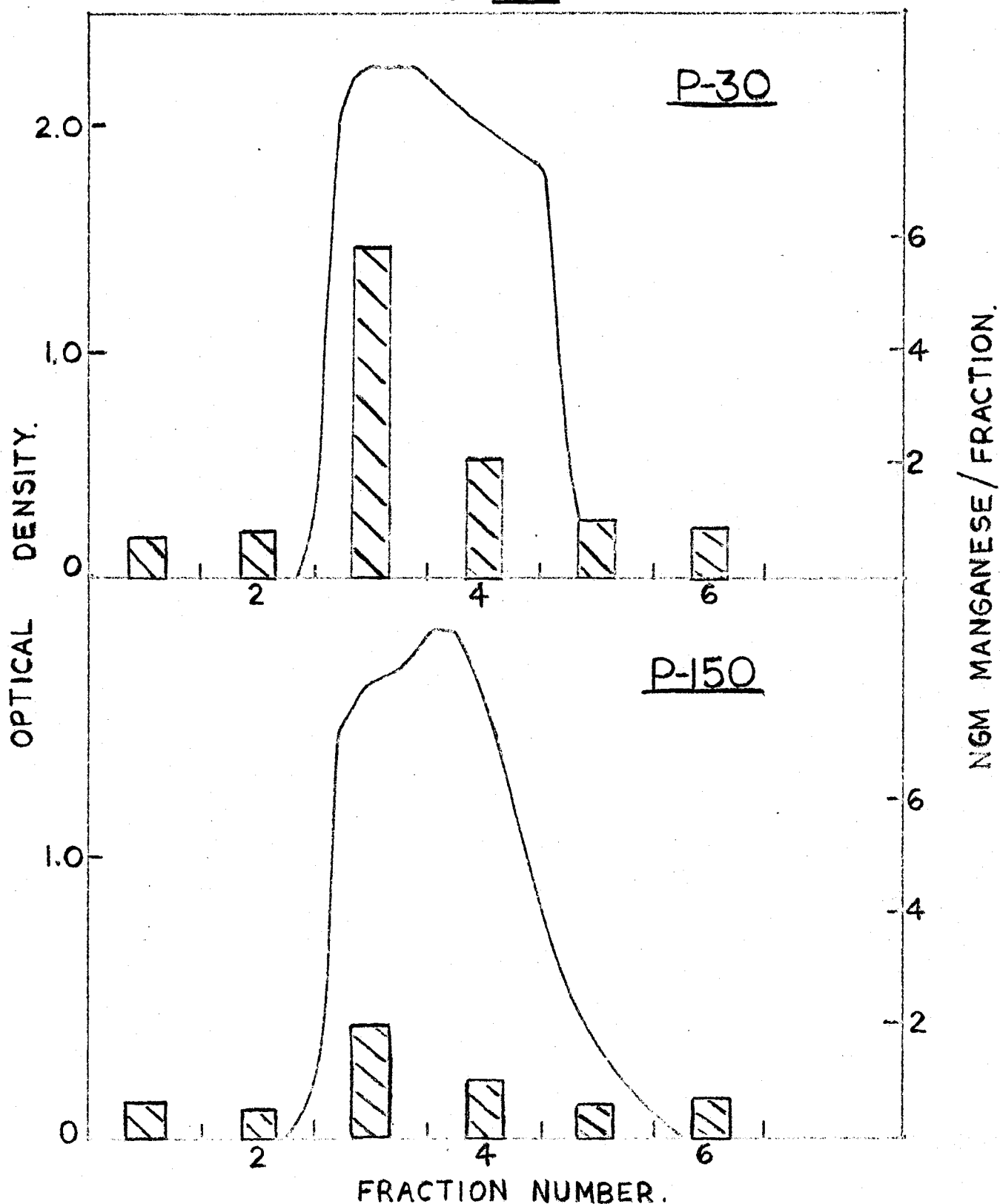
From the in vitro studies of Fritze and Gietz³², together with Holeysovsk's³¹ findings concerning the lability of manganese-albumin complexes it appeared that minor in vitro contamination of the serum samples prior to fractionation might not be too significant. However, the analytical blank for each sample was important, and even by scrupulous purification of reagents and column cleansing it appeared impossible to consistently hold this blank much below 1 ngm/sample.

Another complication arose from the levels of manganese in rabbit serum. Total manganese was of the order of 4 ngm/ml, and when sera were desalted on P-2,0 to P-150 columns the amount of protein-bound manganese appeared to vary depending upon the degree of protein separation and the length of the chromatographic column. This effect may be seen in Fig. VIII. When fractionation was attempted on a large P-200 column the protein resolution in the chromatogram was excellent, as can be seen in Fig. IX, but every fraction contained 1 - 1.3 ngm manganese with no discernible distribution pattern. In this experiment 5 ml serum was fractionated and this should have contained at least 2 ngm protein-bound manganese. It would have been expected that at least two or three adjacent fractions would have appeared above the general background, but this was not observed.

The best results for the activation analysis manganese experiments were obtained in the run pictured in Fig. X, where ~0.5 ml rabbit serum was fractionated on a 34 x 1.8 cm P-200 column and seven 3.5 ml fractions were collected. However, in the light of the previous results no definite inferences could be drawn regarding the distribution of protein-bound manganese in rabbit serum.

Another method of attacking this problem was clearly necessary so it was decided to attempt the in vivo labelling of serum proteins with ⁵⁴Mn. The major problem here arose from previous studies by two groups^{34,36} who had shown that injected ⁵⁴Mn was very rapidly cleared from the blood. Consequently a large dose of the radioisotope was necessary to study any traces which might become associated with the protein.

FIG VIII



1 ml rabbit serum applied to (a) Bio-Gel P-30, (b) Bio-Gel P-150 (#50243) columns each 18 x 1.6 cm. Fraction sizes 5 ml. Manganese in eluate determined by neutron activation analysis.

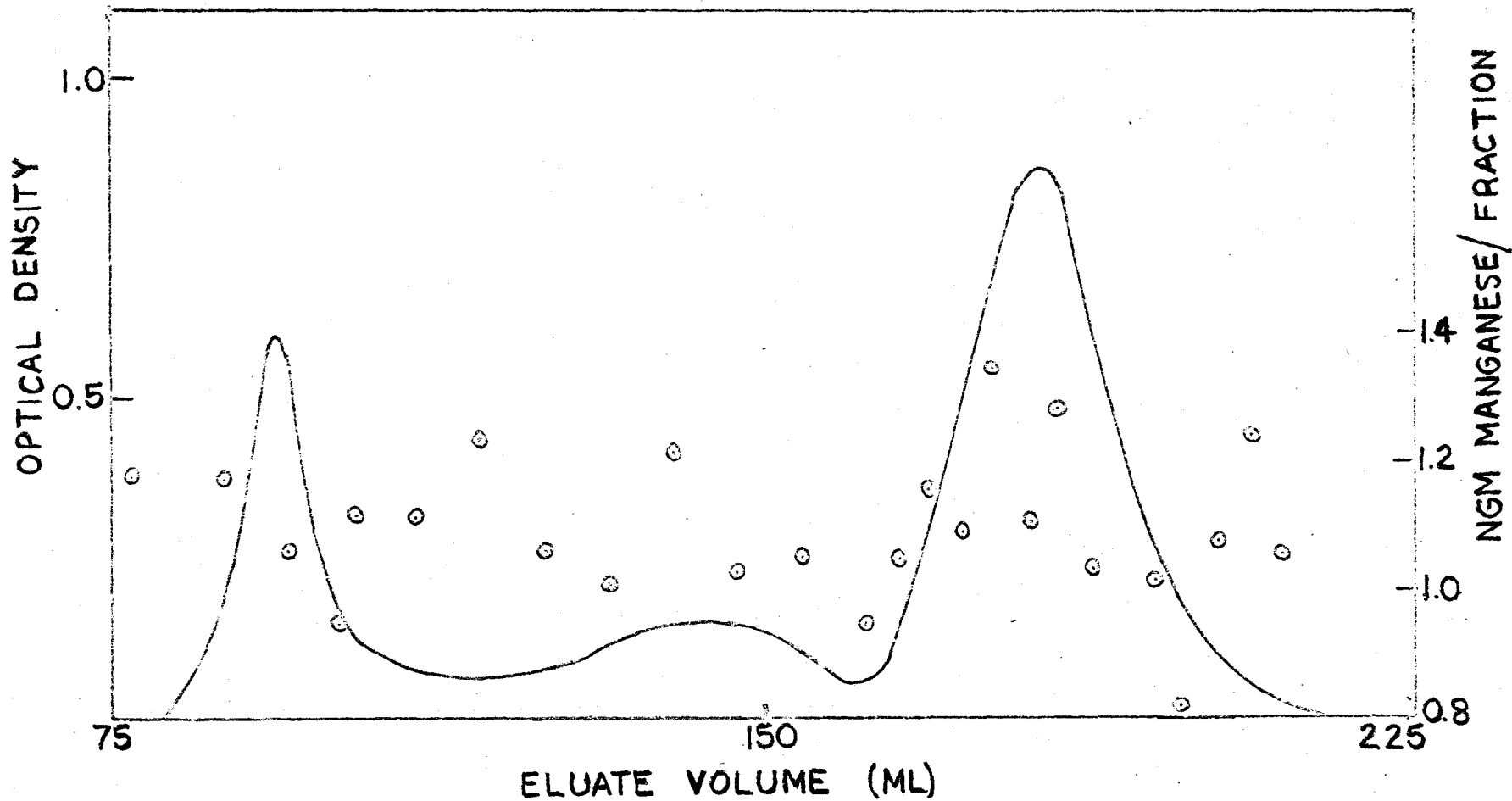


FIG IX

5 ml rabbit serum applied to Bio-Gel P-200 (#47493) column 58 x 3 cm. Fraction size ~4 ml. Manganese in eluate determined by neutron activation analysis.

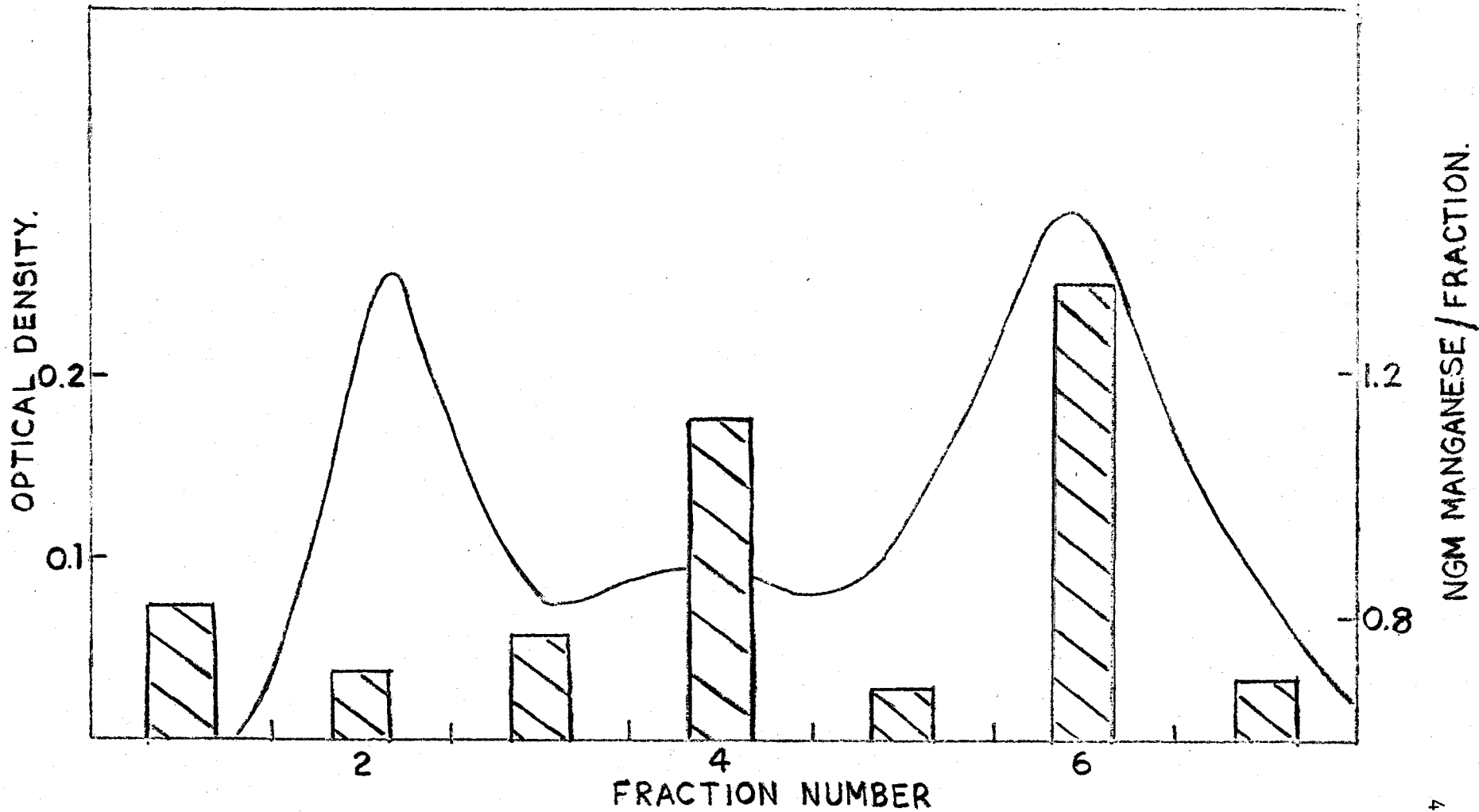


FIG X

0.5 ml rabbit serum applied to Bio-Gel P-200 (#47493) column 34 x 1.8 cm. Fraction size 3.5 ml. Manganese in eluate determined by neutron activation analysis.

In this experiment two rabbits were each injected intravenously with 1 mc of carrier-free ^{54}Mn in sterile physiological saline. This dose in a rabbit corresponds to approximately 30 mc in a human being. Thus, compared with the 2.5 μc per human subject used by Mahoney and Small⁴¹ an increase of 10^4 was obtained in sensitivity for this rabbit experiment. Since ^{54}Mn is a pure γ -emitter the danger of radiation damage to the animal from such a large dose is minimised. Although such large doses of carrier-free manganese may result in temporary upset of manganese metabolism due to disturbance of normal blood levels for the element, these experiments were aimed at studying the longer term distribution of manganese in serum. Hence, in view of the rapid clearance of injected manganese from the blood it was felt that any imbalance would have been righted by the time blood samples of significant interest were taken a week or more after injection.

Blood samples from the dosed rabbits were taken at 12 minutes, 5 days, 7 days, 14 days, 28 days and 93 days after injection of the radioisotope. Generally plasma rather than serum was studied, since the erythrocytes were required for other work (see later).*

Serum from the 12 minute blood sample was subjected to gel chromatography as follows:

(a) 1 ml serum was desalted on P-30 col. 16 x 1.8 cm. This column had been treated with $\text{NH}_2\text{OH}\cdot\text{HCl}$ to dissolve MnO_2 then extensively washed with ultra-pure 0.15 M ammonium acetate solution.

50% of the total ^{54}Mn remaining in the serum appeared to be protein-bound.

* Small plasma and serum samples were counted at various stages of this work, and at no time was there any significant difference in their ^{54}Mn activity.

(b) 1 ml serum was fractionated on P-150 col. 35 x 1.8 cm which had not been specially cleaned. No ^{54}Mn activity was found in the protein region. All was eluted near the total column volume.

(c) 6 ml serum was fractionated on a P-200 col. 58 x 3 cm cleaned as in (a). The results are shown in Fig. XI. It can be seen that all the protein-bound ^{54}Mn occurred in the albumin region of the chromatogram and that this represented $\sim 30\%$ of the total serum ^{54}Mn .

These results implied that upon injection of the ^{54}Mn only a protein similar in size to albumin complexed the isotope, but the complex so formed was labile and the bound ^{54}Mn readily exchanged with inactive manganese in the protein environment, either in the eluent or in the gel material.

Serum samples taken five days after injection of the radioisotope were also chromatographed on the cleaned P-30 column and the cleaned P-200 column used in (a) and (c) above. At this stage the P-30 desalting still showed approximately 50% of the total serum ^{54}Mn to be protein-bound. However, the P-200 fractionation showed a considerable difference from the 12 minute run, as can be seen in Fig. XII. Some of the ^{54}Mn ($\sim 10\%$) was definitely incorporated into a region of the protein chromatogram corresponding to a molecular size between that of the high molecular weight peak and the γ -globulin peak. If this manganese compound is a globular protein, it would appear to have a molecular weight of about 200,000. At the 5-day stage about 10% of the total serum ^{54}Mn activity occurred in this peak, and less than 5% was in the albumin region. Of the remaining activity about

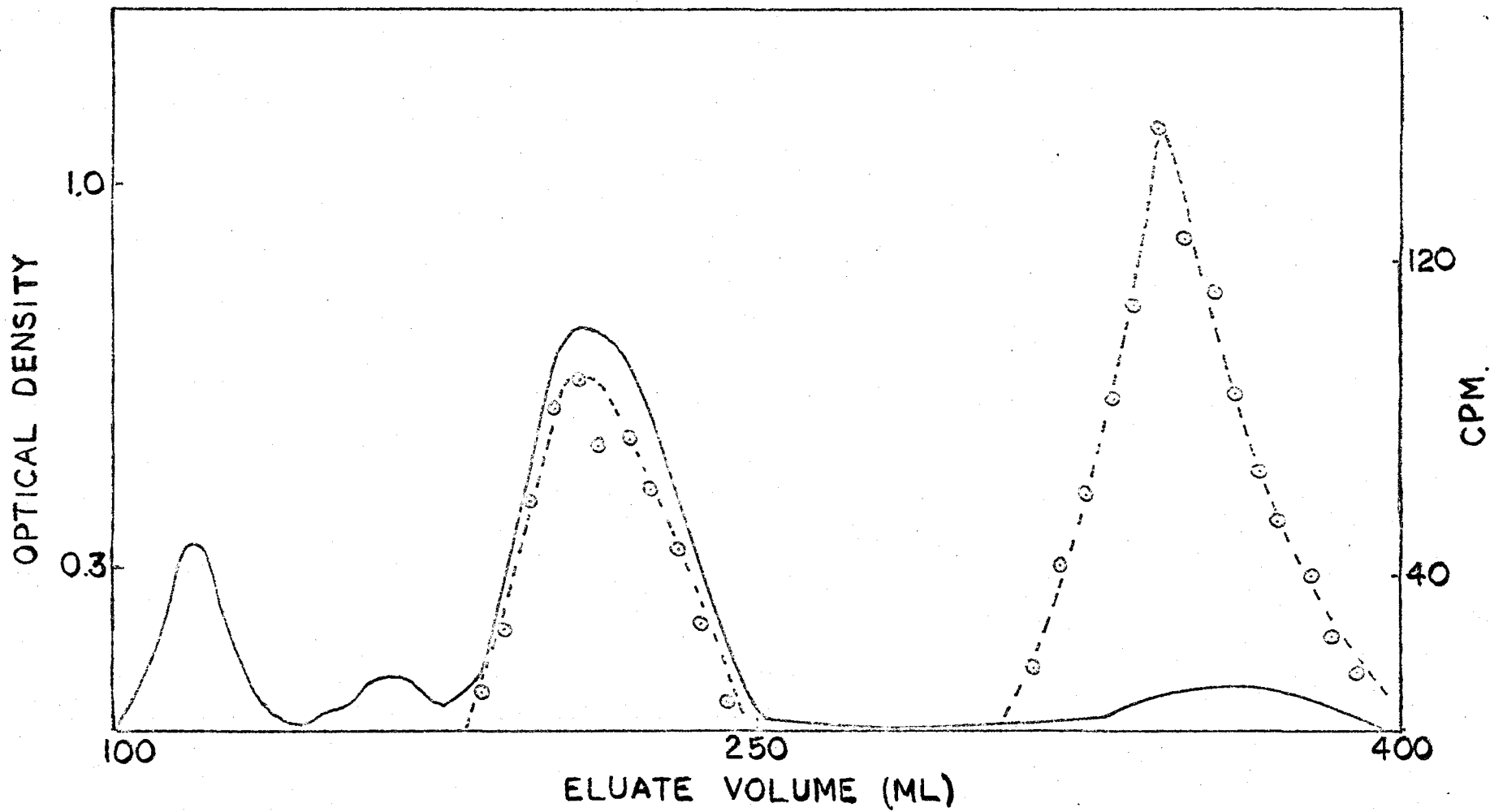
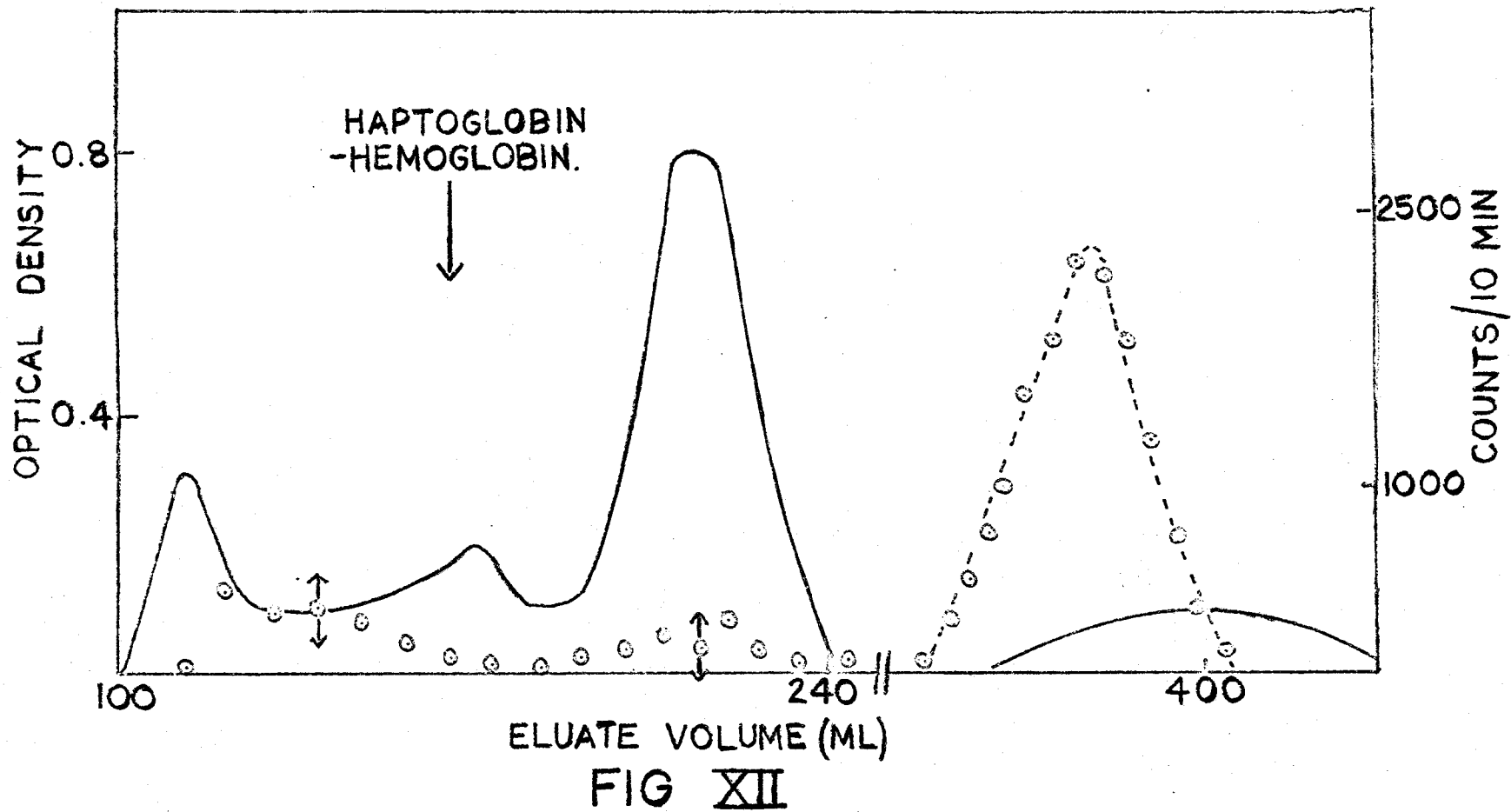


FIG XI

8 ml rabbit serum applied to Bio-Gel P-200 (#47493) column 58 x 3 cm. Fraction size 6 ml. Sample obtained 12 min. after injection of rabbit with ~1 mc carrier-free ⁵⁴Mn.



10 ml rabbit serum applied to Bio-Gel P-200 (#47493) column: 58 x 3 cm. Fraction size 10 ml. Sample obtained 5 days after injection of rabbit with ~1 mc carrier-free ⁵⁴Mn.

10% lay between the albumin and the ionic region of the chromatogram and 75% was eluted at a very low molecular weight position just ahead of the chloride region (detected by addition of AgNO_3).

After seven days 15% of the total activity appeared in the high molecular weight fraction of plasma, 7% in the albumin region, and approximately 75% was still apparently ionic. Only 3% of the ^{54}Mn appeared between the albumin and ionic region.

At 14 days after injection the total protein-bound ^{54}Mn activity amounted to ~75% of the total plasma activity.

By 28 days after administration the ^{54}Mn levels in plasma had fallen considerably but there was still sufficient activity to show that when 10 ml plasma was fractionated on the P-200 column 58 x 3 cm 37% of the ^{54}Mn occurred in the protein fraction corresponding to a molecular weight of approximately 200,000. Less than 1% lay between this peak and the albumin region where another 35% of the ^{54}Mn occurred. Only 19% of the activity appeared in the ionic region and 8% between the albumin and ionic areas. These results are shown in Fig. XIII in two ways. The ^{54}Mn count rate in each fraction was very low and most of the fractions gave only the background count. The two horizontal lines represent the standard deviation of these fractions. It can be seen that in two parts of the protein spectrum the count rates of adjacent fraction lay well outside these lines, and these regions represent the 200,000 and albumin-bound manganese. The shaded bars in Fig. XIII are the count rates of the combined fractions in the indicated regions.

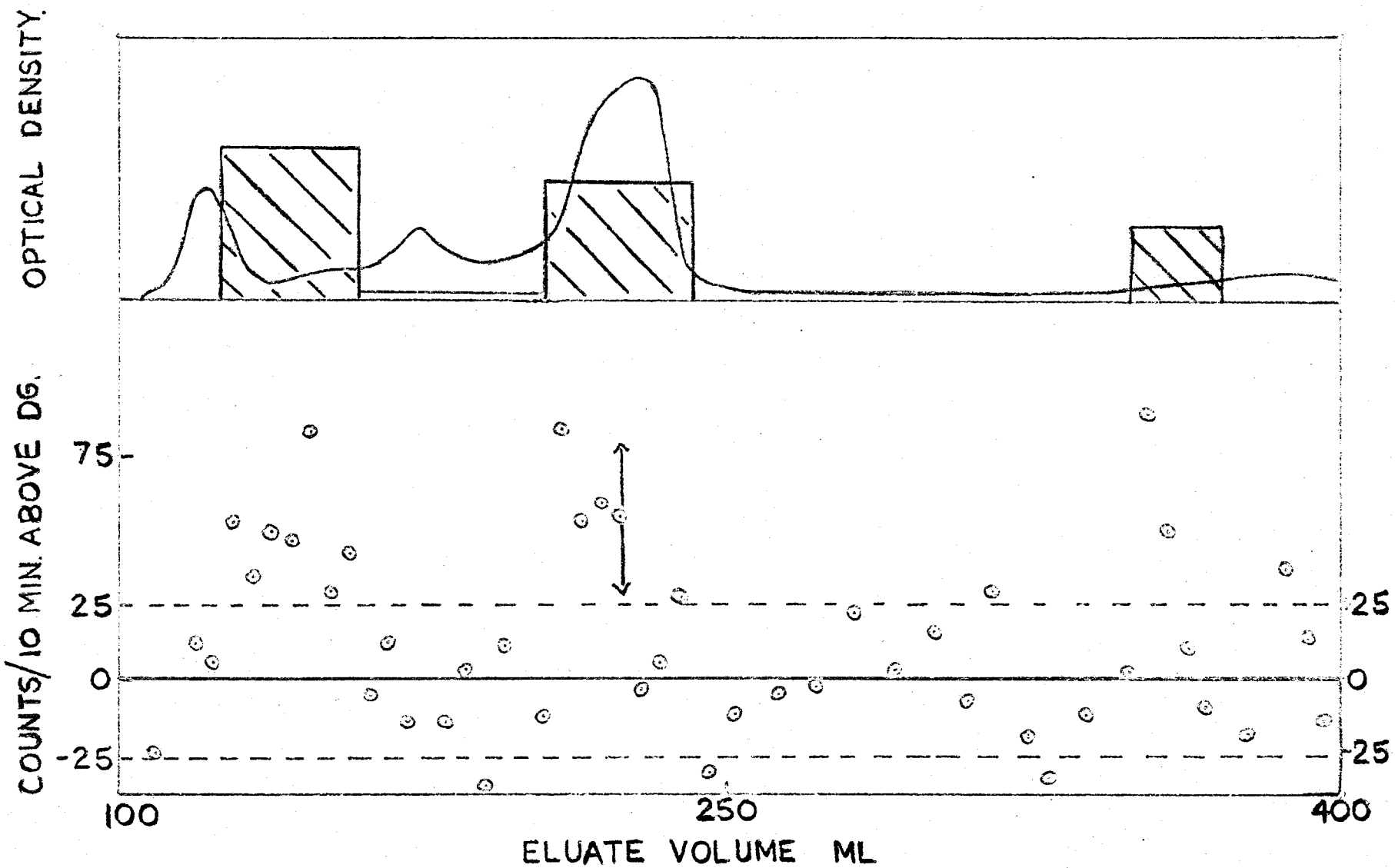


FIG XIII

10 ml rabbit plasma applied to Bio-Gel P-200 (#47493) column: 58 x 3 cm. Fraction size 6 ml. Sample obtained 31 days after injection of rabbit with ~1 mc carrier-free ⁵⁴Mn.

Three months after administration of the ^{54}Mn there was only 4.8 ± 0.3 c/min/ml plasma, above a room background of 65 cpm and after fractionation of 10 ml of this material over the large P-200 column used previously, no activity could be detected in the eluate.

The experiments described above represent the first time the incorporation of ^{54}Mn into serum proteins has been observed in vivo so long after administration of the isotope. The $\sim 200,000$ molecular weight compound into which the ^{54}Mn is incorporated with a relatively long half-life was at first attributed to transmanganin. (However see p. 75.) Foradori et al.³⁰ showed that when human serum treated in vitro with ^{54}Mn was subjected to electrophoresis, virtually all the radioactivity migrated with the globulin fraction, and more specifically with β_1 -globulins. The same results were obtained using rat serum, labelled in vivo with ^{54}Mn , 4 hours prior to blood sampling.

Since the rabbit serum/plasma experiments showed that soon after injection of ^{54}Mn the only protein-bound ^{54}Mn occurred in the albumin region of the chromatogram, an experiment involving in vitro addition of carrier-free ^{54}Mn to rabbit serum was performed. The radioisotope and 2 ml serum were incubated for 30 minutes at 37°C and then applied to a P-200 column. The elution pattern is shown in Fig. XIV. Almost 50% of the added activity was found in the albumin region of the chromatogram. This was followed by a flat region containing 14% of the activity, an activity peak representing another 13% and finally an ionic peak containing the remaining ^{54}Mn .

Consequently it may be inferred that manganese may be bound by at least two proteins in serum. The lower molecular size manganese-binding protein which is eluted from a gel chromatographic column in

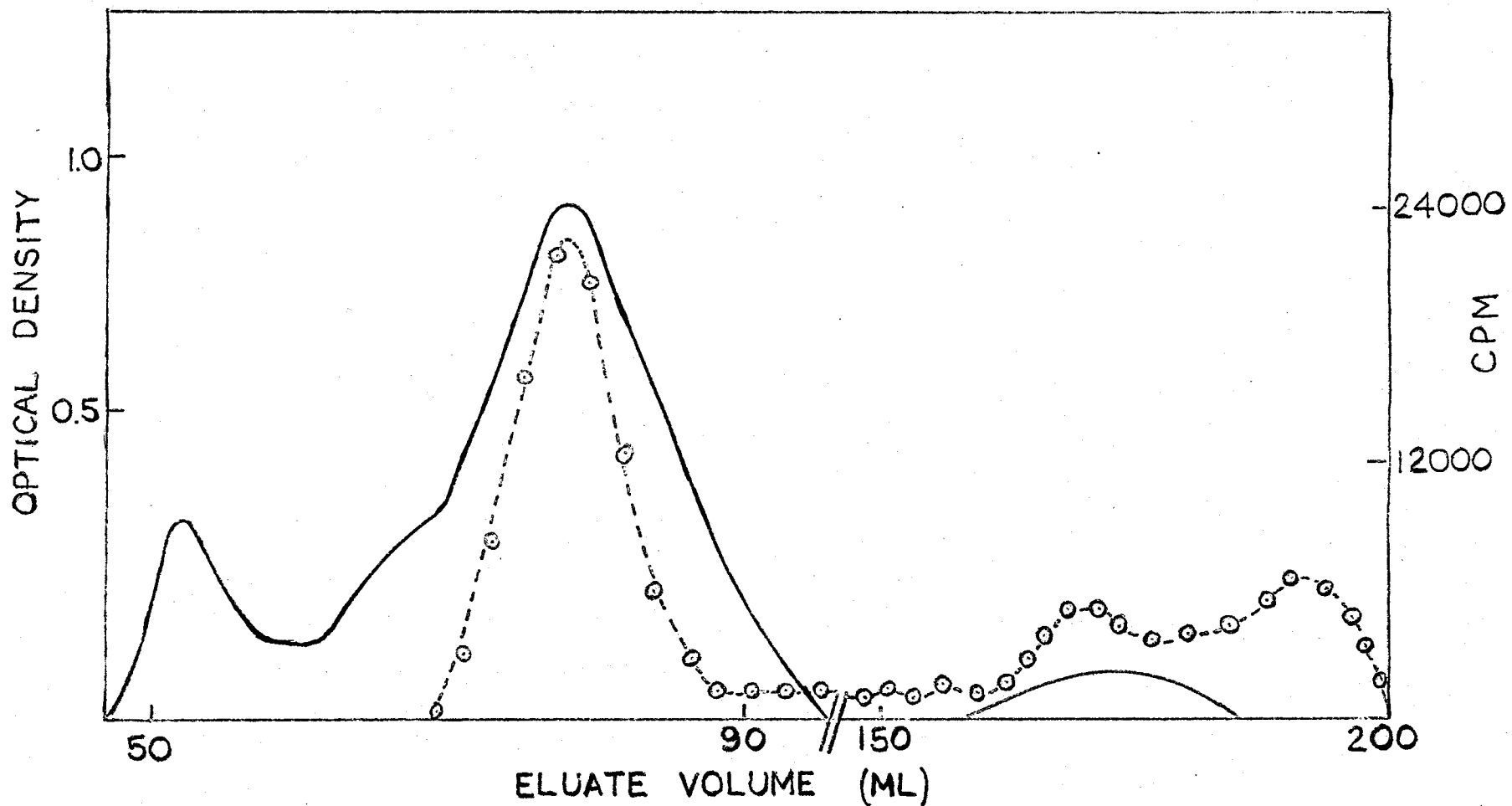


FIG XIV

2 ml rabbit serum + carrier-free ^{54}Mn applied to Bio-Gel P-200 (#47493) column
 40 x 2.4 cm. Fraction size ~3 ml. Serum + isotope incubated for 30 min.
 before application to column.

the same position as albumin appears to function as a carrier only, since the complex formed is relatively labile. If this was an albumin complex, it would agree with Holeysovska's findings concerning the lability of the manganese-albumin complex. However, this would conflict with the work of Foradori et al. who not only found virtually no in vivo or in vitro association between albumin and manganese, but also showed by paper electrophoresis that added ⁵⁴Mn migrated with a β_1 -globulin.

These apparently conflicting results may be reconciled by assuming that the manganese binding component observed in gel chromatographic studies in vitro is not albumin, but some other protein of similar molecular size to albumin. This is almost certainly the β_1 -globulin reported by Foradori et al., since Tozer and Pirt⁴⁶ have shown by electrophoresis that although the third peak obtained by fractionation of serum on Sephadex G-200 consists mainly of albumin, some α - and β -globulins are also eluted in this region. If this were the case, the very low apparent albumin binding capacity would be explained. It seems unreasonable that albumin, which represents more than 75% of the serum proteins (~ 350 mgm/ml) should be able to bind only the minute (~ 1 ngm/ml) quantities of manganese suggested by the results of Fritze and Gietz. This point has previously been made with respect to the albumin copper binding capacity⁴⁹, and it could well be that in this case also, some other protein than albumin is responsible for the metal binding, as appears to be the case for manganese.

The higher molecular weight manganese protein, detected for the first time in these studies, presumably has some metabolic

function other than manganese binding, since it only appears in the serum labelled with ^{54}Mn after an extended time period. This suggests that the protein is being synthesized with the incorporation of ^{54}Mn in a manner perhaps similar to the hepatic synthesis of ceruloplasmin.

Since other work (see later) showed that erythrocytic manganese exists as a porphyrin bound to globin in a compound analogous to hemoglobin, it was necessary to check that this higher molecular weight compound was not perhaps a haptoglobin-globin-manganese porphyrin complex similar to haptoglobin-hemoglobin. Tests on the particular rabbit serum used, by means of the benzidine/ H_2O_2 ⁵¹, test for hemoglobin showed the latter to be eluted from a P-200 column at a lower molecular size position than the high molecular weight manganese protein. The elution position of the haptoglobin-hemoglobin complex is marked with an arrow in Fig. XII.

Manganese in Erythrocytes

Since Borg and Cotzias³⁴ had shown that ^{54}Mn intravenously injected into animals disappeared from the bloodstream very quickly and then reappeared much more slowly in the erythrocytes, it was decided to study manganese in erythrocytes using the radioactive tracer. Although the manganese levels in erythrocytes are higher than in serum, the large quantities of iron, present as hemoglobin, could well have interfered with an application of the neutron activation analysis method developed for the study of copper distribution in serum, by the reaction $^{56}\text{Fe}(n,p)^{56}\text{Mn}$. Other workers^{28,33} have shown that this reaction accounted for 30% of the ^{56}Mn produced in analyses for total manganese in serum.

As the incorporation of ^{54}Mn into erythrocytes clearly takes place well after administration of the isotope, it seems unlikely that studies by this method run too much danger of producing misleading results by alteration of the manganese balance, particularly if the carrier-free isotope is used.

A rabbit was injected with 1 mc ^{54}Mn (carrier-free) and blood samples were taken as required, after allowing several days for reappearance of the isotope in the erythrocytes. The rate of reappearance was not measured since this is well documented elsewhere.^{34,35}

Hemolysates were prepared from the washed erythrocytes as detailed in the experimental section (d). Gel chromatography of the hemolysate on Bio-Gel P-150 produced the chromatogram shown in Fig. XV. 1.5 ml hemolysate was applied to a column 45 x 1.8 cm, and as can be seen, virtually all ($> 95\%$) of the ^{54}Mn activity occurred in a single peak which lined up almost exactly with the single protein peak. This latter peak represents the hemoglobin content of the hemolysate. By virtue of its preponderance as the protein content of erythrocytes hemoglobin is the only material detected by the absorptiometer monitoring the eluate from the chromatographic column. A small ^{54}Mn peak was also observed at an elution volume corresponding to that for ionic material.

Hemin was prepared from the hemolysate essentially by the method of Lewis⁴⁷. The hemolysate was acidified to pH = 2 with HCl solution and the protein precipitated by addition of acetone. The precipitate (mainly globin) was centrifuged off, and the acetone evaporated from the supernatant hemin solution. Hemin precipitated on removal of acetone was centrifuged off and redissolved in the

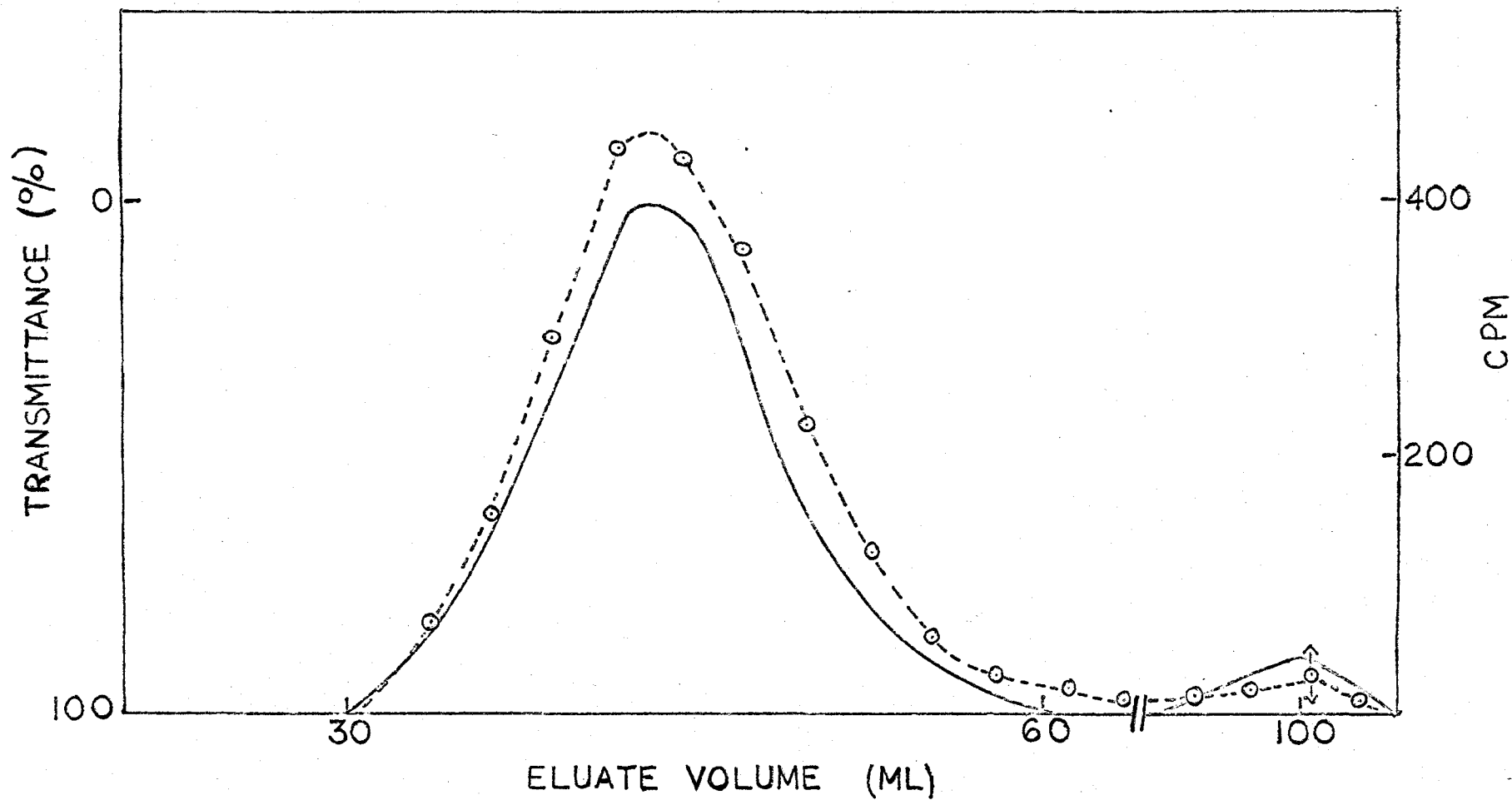


FIG XV

1.5 ml rabbit hemolysate labelled in vivo with ^{54}Mn applied to Bio-Gel P-150 (#50243) column 45 x 1.8 cm. Fraction size 6 ml.

minimum volume of acetone or dioxane. This solution was chromatographed on a column of Bio-Beads SX-8 using acetone as eluent.

In a typical hemin preparation the distribution of ^{54}Mn was as follows (all count rates corrected to the same volume):

Protein Precipitate 106 ± 3 cpm

HCl supernatant (after evaporation of
acetone and precipitation of hemin) 175 ± 4 cpm

Hemin solution in acetone 1395 ± 6 cpm

Hence more than 80% of the original erythrocyte ^{54}Mn was recovered in the final acetone solution of hemin.

Fractionation of hemin solution on Bio-Beads SX-8 is shown in Fig. XVI. The elution of the hemin was followed by measurement of the eluate absorption at 400 m μ , using a Spectronic 20 spectrophotometer. It can be seen that there was a slight separation of hemin and the ^{54}Mn -binding compound. This separation was not reproducible, occurring in four out of the eight hemin fractionation runs performed. In the other four experiments no separation was observed.

In the cases where separation occurred the ^{54}Mn compound always appeared at a lower molecular size position than the hemin. The ^{54}Mn was never eluted ahead of the hemin, which in turn was always eluted with a lower apparent molecular size than chlorophyll, which was used as a standard on these columns. The four runs in which separation occurred were, with one exception on newly packed columns, and in these cases there was some adsorption (up to 50%) of the ^{54}Mn compound on the column. In the no-separation runs the chromatographic columns had been used previously, and there was generally less adsorption of ^{54}Mn in these experiments.

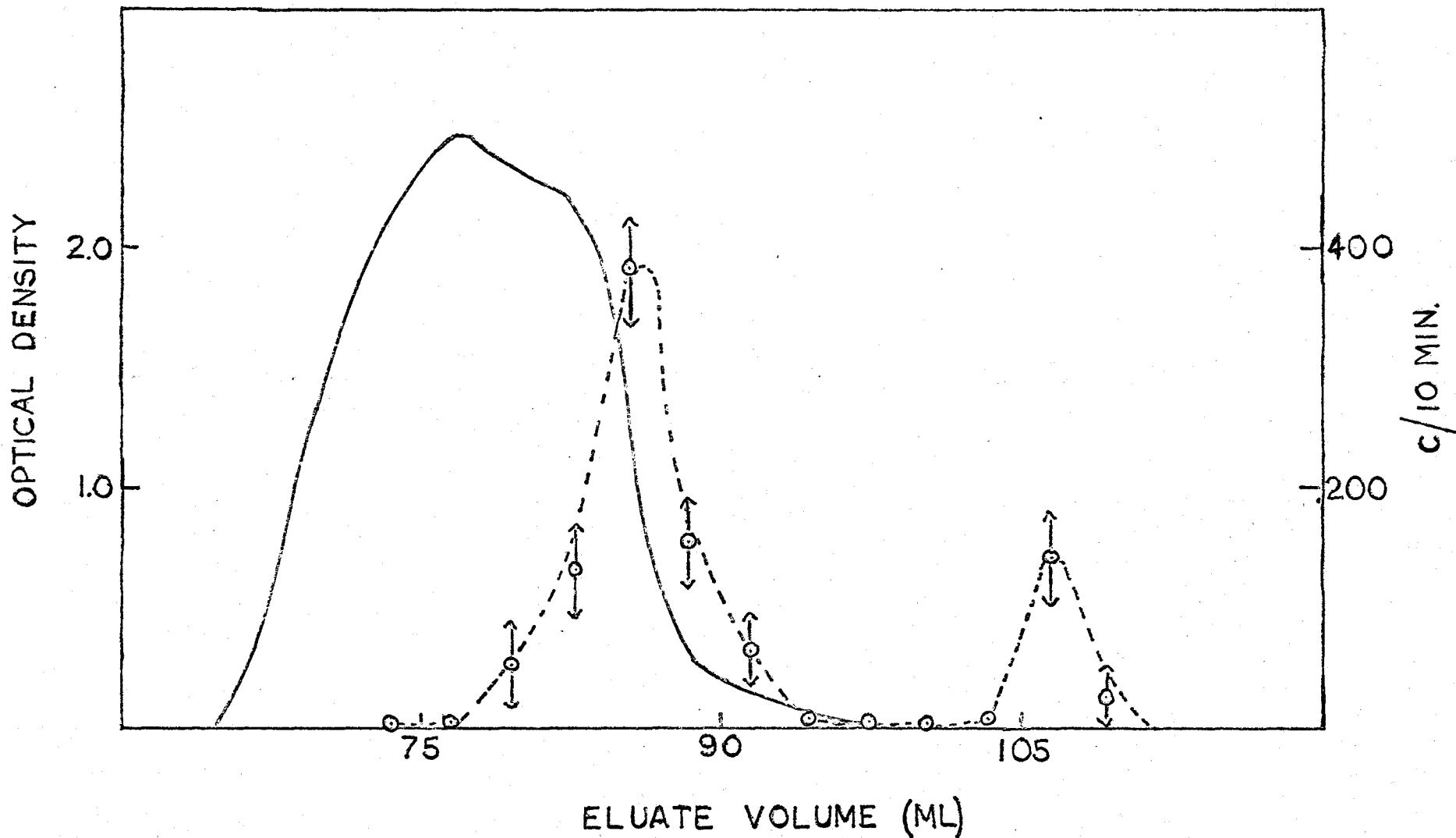


FIG XVI

Hemin solution in acetone (ex *in vivo* ⁵⁴Mn-labelled erythrocytes) applied to Bio-Bead SX-8 column 65 x 1.8 cm. Eluent acetone. Optical density of eluate monitored at 400 m μ .

In all eight fractionations a small ^{54}Mn activity peak was detected at an elution volume corresponding to $\sim 75\%$ of the column volume. The elution parameters for Bio-Beads in acetone are not yet established in the literature, but experiments with ^{24}Na dissolved in $10\% \text{H}_2\text{O}/\text{CH}_3\text{COCH}_3$ (1:10) showed that small ions are eluted at approximately 80% of the column volume under the conditions used in these studies.

Addition of alkali to hemin results in polymerization of iron porphyrins⁴⁸, and this effect was observed when alkaline hemin solutions were fractionated on Bio-Gels with low exclusion limits (Fig. XVII). This figure shows the results of chromatographing an alkaline hemin solution on P-4, using $0.1 \text{ M NH}_4\text{OH}$ solution as eluent. It can be seen that the ^{54}Mn activity occurs with the hemin polymer peak. It appears that under the conditions used here the hemin polymer has a molecular weight of the order of 10,000 since its elution volume on a P-10 column is slightly greater than the elution volume of Blue Dextran (M. wt. $\sim 2 \times 10^6$). An accurate assessment of the hemin polymer molecular weight is not possible and this figure (10,000) must be regarded as a minimum since the carbonamide linkages in polyacrylamide gels are subject to hydrolysis in media of $\text{pH} > 9^5$. This effect became increasingly pronounced the higher the exclusion limit gel used and rendered the use of gels with larger pore sizes than those in P-10 impracticable since the continuous swelling of the gel clogged the column before any experiment could be completed. However, the important result is that the ^{54}Mn compound was also polymerized and eluted with the hemin polymer in

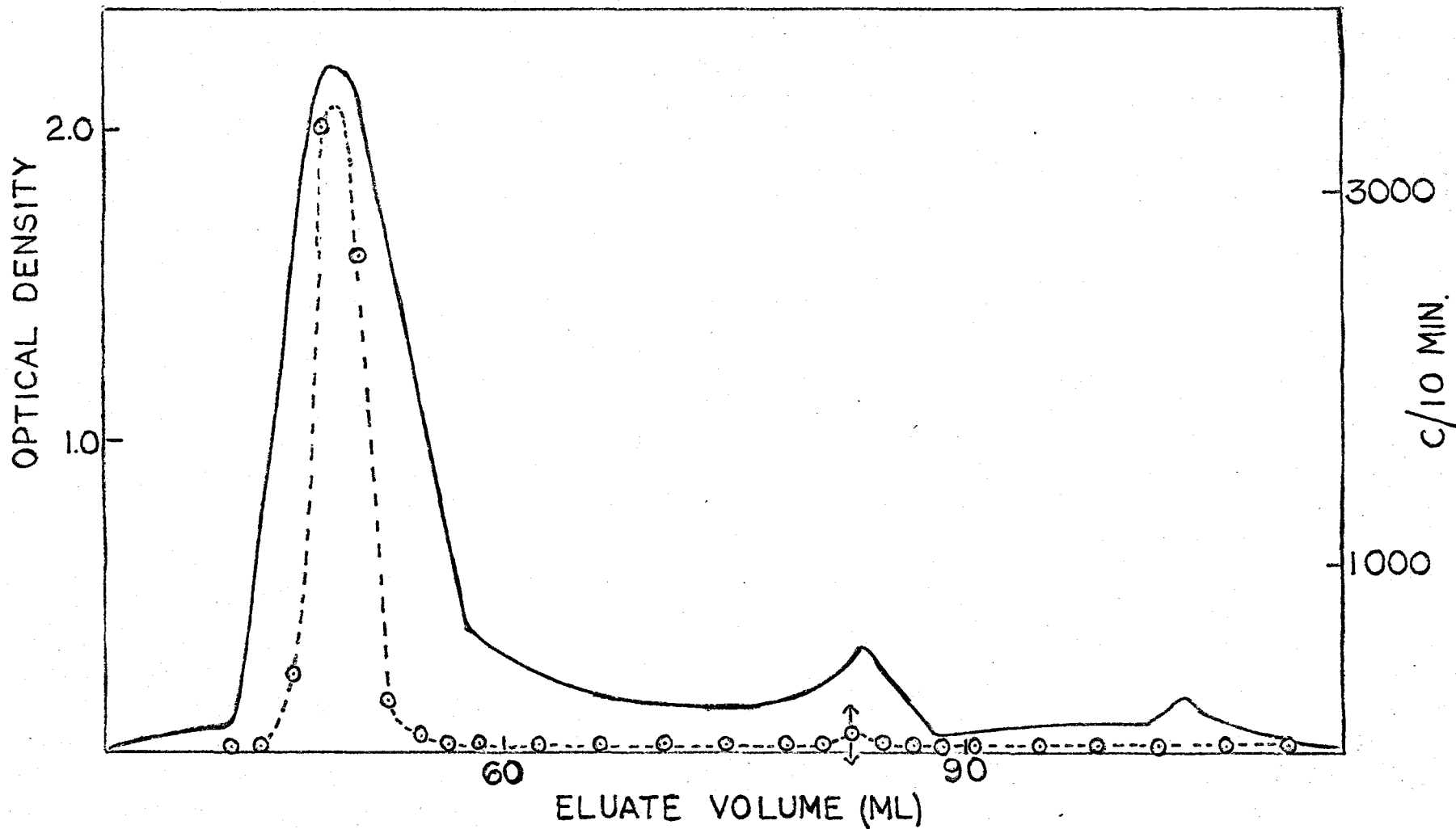


FIG XVII

Hemin solution in 0.1 M NH_4OH (ex in vivo ^{54}Mn -labelled erythrocytes) applied to Bio-Gel P-4 column 50 x 1.8 cm. Eluent 0.1 M NH_4OH . Eluate optical density monitored at 253 μ .

all cases. The polymerization of manganese porphyrins has not previously been observed⁴⁸. A lower molecular size peak (molecular weight ~ 500) also contained a small amount of ^{54}Mn activity and was attributed to the monomeric units since this fraction had an absorption maximum for radiation of wavelength $\sim 400 \text{ m}\mu$ (characteristic of iron porphyrins). Another peak at a still lower molecular weight was shown by a blank run to be due to an impurity in the reagent grade acetone used to extract the hemin from the hemolysate.

These results all strongly support the Berg and Cotzias hypothesis for the existence of a naturally-occurring manganese porphyrin in erythrocytes. From gel chromatography of hemolysates it appears that the porphyrin is bound to globin to form a manganese analogue of hemoglobin and that virtually all of the erythrocytic manganese is in this form. Gel chromatography of hemin solutions in acetone showed that the ^{54}Mn compound which was removed from the hemolysate under classical porphyrin extraction conditions has a molecular size similar to that of hemin, therefore providing even more convincing evidence for the existence of a manganese porphyrin.

Copper in Erythrocytes

In an attempt to investigate the in vivo distribution of copper in erythrocytes, a human hemolysate sample was fractionated on an "ultra-clean" (see Experimental section) P-200 column. Each fraction was analysed for copper by neutron activation analysis, and the results are shown in Fig. XVIII. All of the protein-bound copper was eluted in a single peak occurring shortly after the hemo-

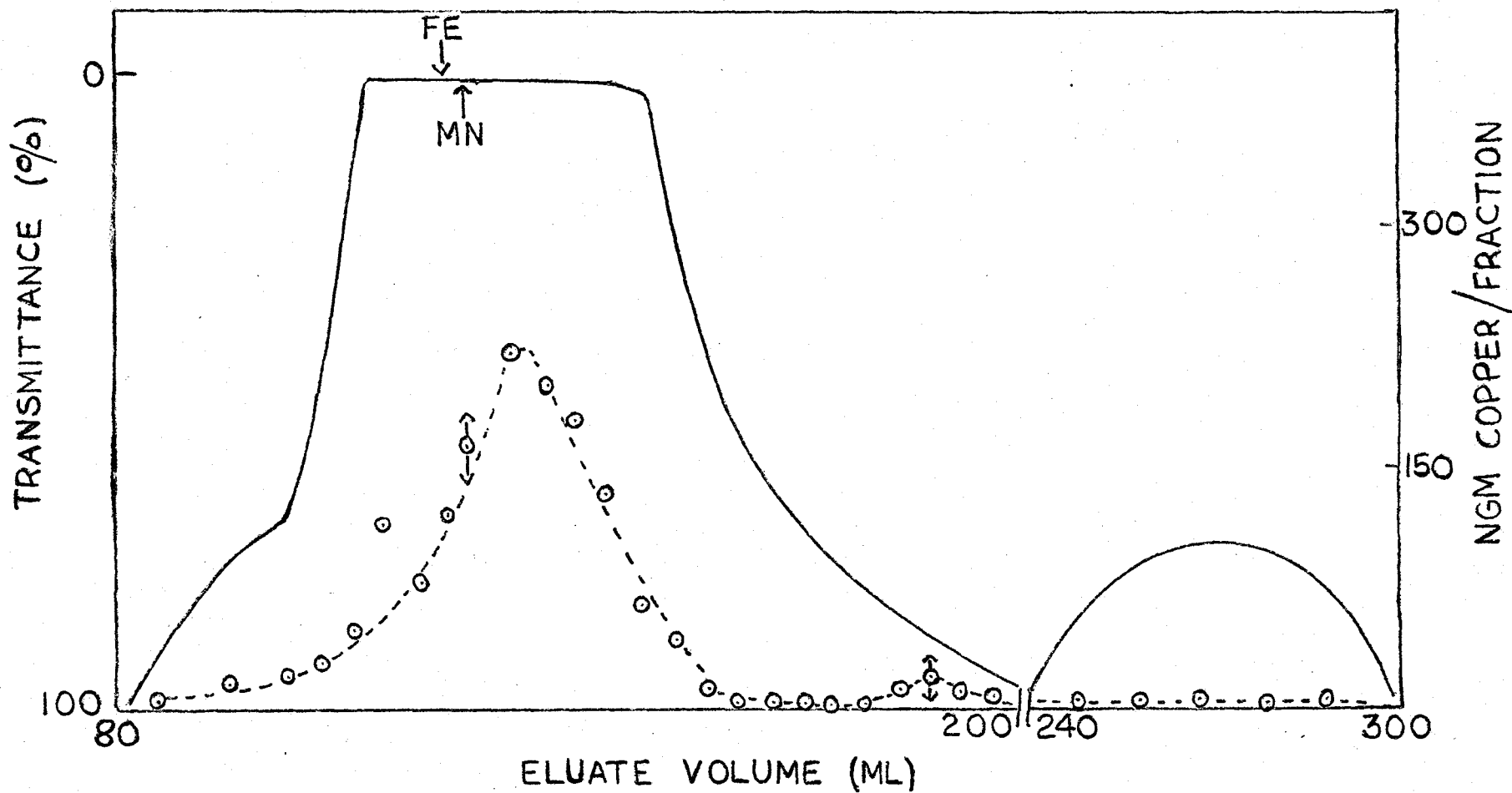


FIG XVIII

3 ml human hemolysate applied to Bio-Gel P-60 column 50 x 2.6 cm. Fraction size 4 ml. Copper in eluate determined by neutron activation analysis.

globin peak. A mass balance showed this contained almost all the copper in the sample, though a small copper peak occurred in an elution position corresponding to a molecular weight of approximately 4000. This peak contained less than 3% of the total copper, and no ionic copper was detected.

During the fractionation of the sample on the gel column the intense hemoglobin absorption on the ultra-violet monitor gave a long flat reading of zero transmission over a considerable portion of the elution curve. Hence the hemoglobin elution peak position could not be measured from the chromatograph but was instead fixed by the ^{56}Mn activity peak in the samples. Since previous work had shown that erythrocyte manganese is eluted on gel columns in the same volume as hemoglobin, the two sources of ^{56}Mn , $^{55}\text{Mn}(n,\gamma)^{56}\text{Mn}$ and $^{56}\text{Fe}(n,p)^{56}\text{Mn}$ did not interfere, and in fact their coincidence made the fixing of the hemoglobin elution maximum more precise. In addition, despite the short irradiation (2 hours) the ^{59}Fe produced by $^{58}\text{Fe}(n,\gamma)^{59}\text{Fe}$ could be detected in the peak hemoglobin fractions. The iron and manganese peaks are marked in Fig. XVIII with arrows.

Although zinc levels in erythrocytes are approximately ten times the copper levels¹⁰ (12 ppm vs. 1 ppm) the same argument about interference in copper analysis by the reaction $^{64}\text{Zn}(n,p)^{64}\text{Cu}$ applies for the erythrocyte situation as it did for serum (p. 31).

Although the mass balance indicated no contamination with copper during the chromatography, the pick-up of ionic ^{64}Cu by hemolysate proteins was studied. Carrier-free ^{64}Cu was added to a hemolysate sample and after a 30-minute incubation period at 37°C. 1 ml

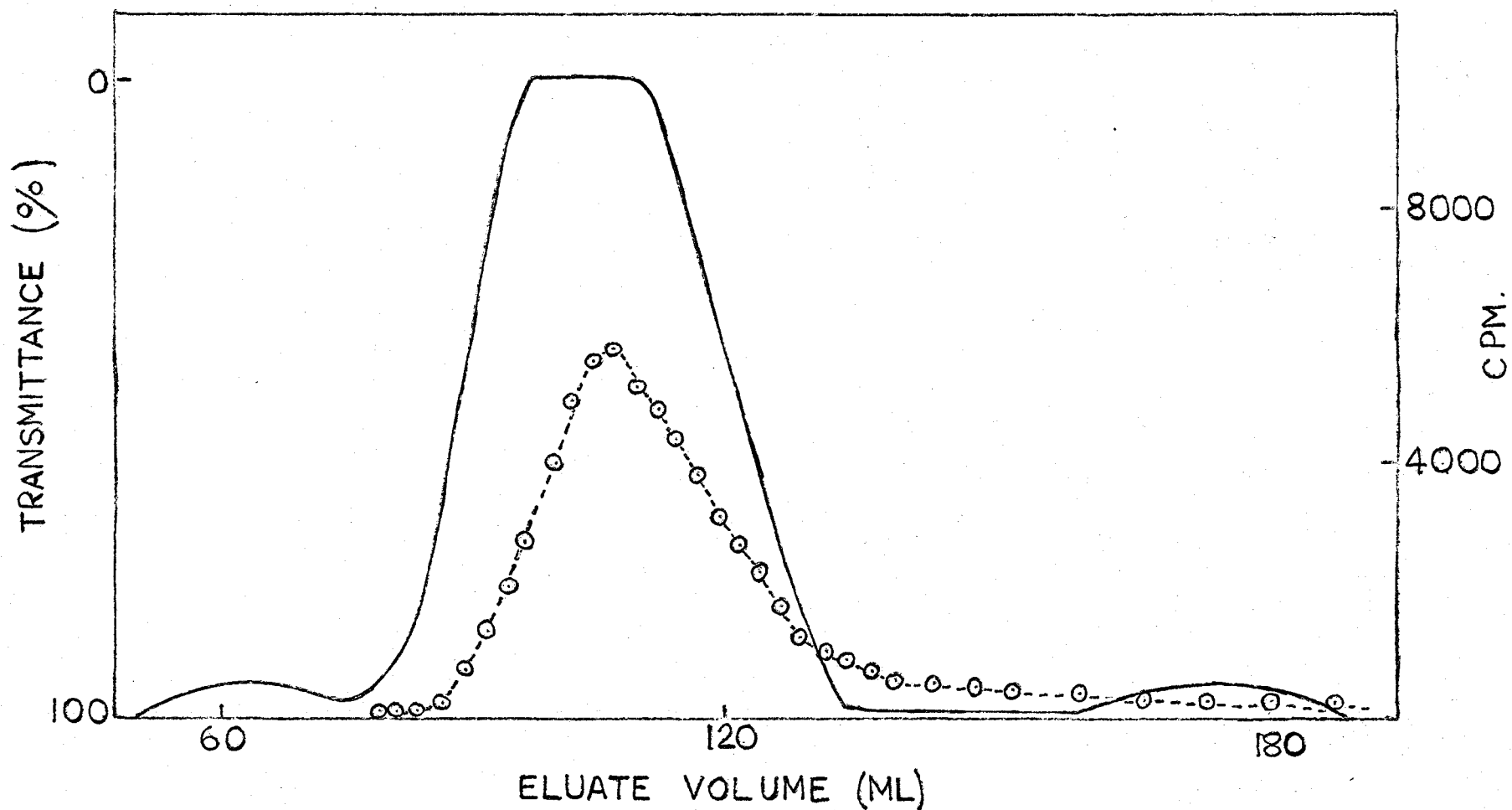


FIG XIX

1 ml human hemolysate + carrier-free ^{64}Cu applied to Bio-Gel P-200 (#47493) column 38 x 2.4 cm. Fraction size 2 ml. Hemolysate + isotope incubated for 4 hr. prior to application.

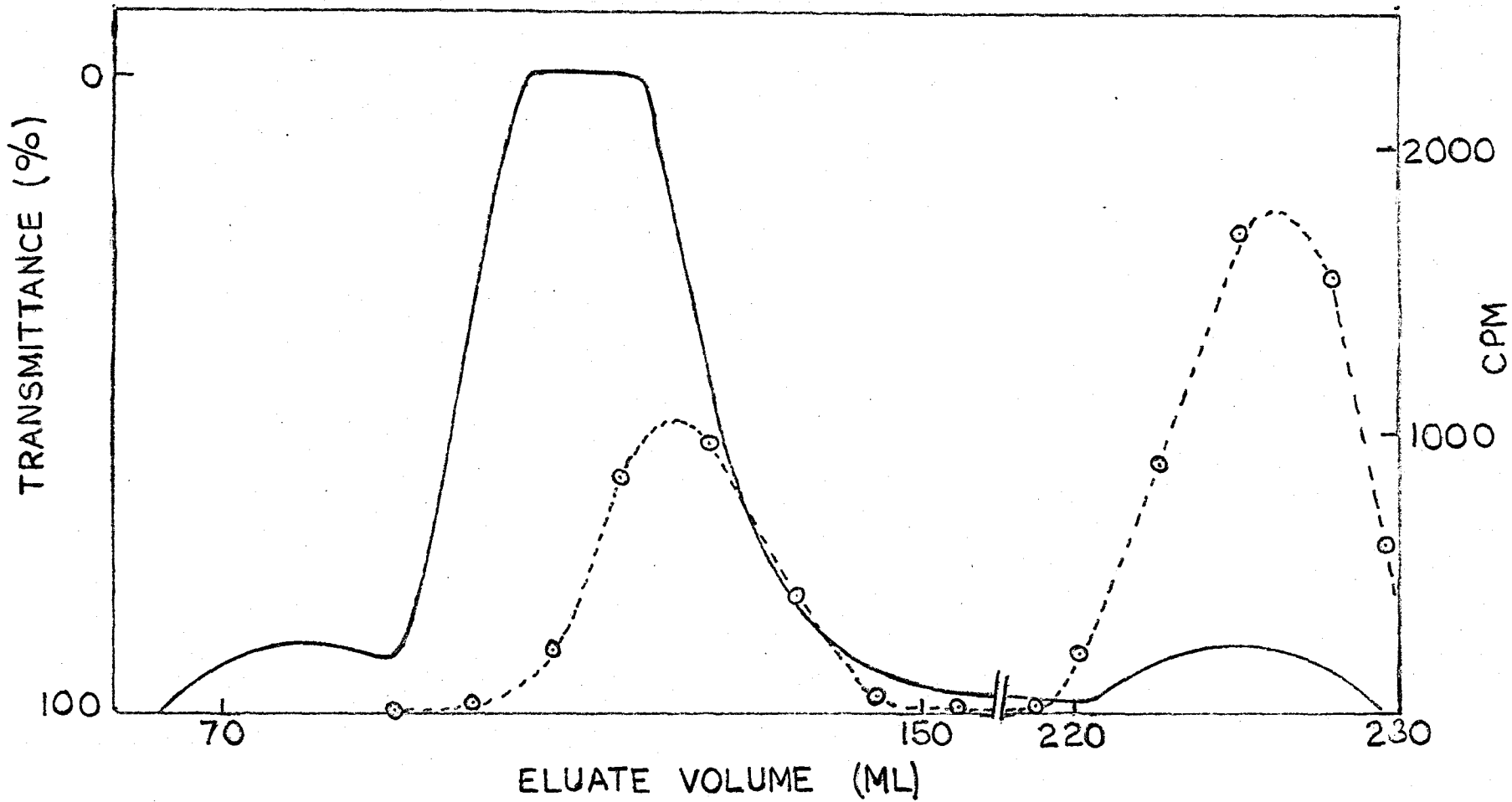


FIG XX

1 ml human hemolysate + carrier-free ^{64}Cu + 1 mgm EDTA applied to Bio-Gel P-60 column 50 x 2.6 cm. Fraction size 8 ml.

of the mixture was fractionated on a P-200 column 38 x 2.4 cm. The ^{64}Cu activity in relation to the protein elution curve are shown in Fig. XIX. It can be seen that virtually all the ^{64}Cu was complexed by the protein, mostly in the hemoglobin region, though there was evidence of a shoulder on the lower molecular weight side of the peak.

Addition of EDTA to the remaining [hemolysate + ^{64}Cu] mixture, followed by 12 hours incubation at 37°C, and fractionation on a P-60 column resulted in Fig. XX. Here ~70% of the ^{64}Cu is eluted in a volume corresponding to an ionic form, and only ~30% is protein bound. However, this 30% is bound in the region where the previous experiment (Fig. XIX) showed a ^{64}Cu activity shoulder, and consequently it appears that the ^{64}Cu activity bound in the hemoglobin region has been removed by the EDTA, but that bound by a protein of a slightly lower molecular size could not be complexed by EDTA.

It should be noted that the data for Fig. XX was obtained ~40 hours after that for Fig. XIX. Hence it was necessary to collect larger, and consequently fewer, fractions. However, if the data is corrected for decay of ^{64}Cu , the total activity involved in each experiment is approximately the same, and in spite of the fewer data points in Fig. XX, the results are quite unambiguous.

A combination of the activation analysis results with the in vitro studies suggests that the protein which binds all the copper in erythrocytes in vivo, also has the capacity to complex more copper in vitro or can exchange readily with ionic copper in solution. Hence based upon these experiments alone the most reasonable assessment of the in vivo copper situation in erythrocytes

would be that virtually all the copper was bound in a single protein complex. Since in vitro addition of extra copper (as carrier-free ^{64}Cu) still shows no evidence of the presence of ionic copper after gel chromatography, the erythrocyte proteins obviously have a copper-binding capacity which enables the complexation of more of the element than is normally present in the red cells.

However, this description is at odds with recent publications on erythrocyte copper. Stansell and Deutsch²⁶ have shown that only 30-50% of red cell copper is bound as erythrocuprein, the remainder being dialysable. Neumann and Silverberg²⁷ also support the hypothesis of Bush et al.⁵⁰ that there are two copper pools in erythrocytes, one labile and dialysable, the other non-labile and bound as erythrocuprein.

While it is difficult to reconcile the experimental results recorded in this thesis with those in the literature, there are some apparent inconsistencies in the latter which are at least worth commenting on.

The original isolation of erythrocuprein²⁴ was performed using an anti-erythrocuprein serum, and the results indicated that erythrocuprein accounted for nearly all the erythrocyte copper. Later experiments showed this erythrocuprein was antigenically inhomogeneous, and subsequent preparations used more highly specific anti-serum^{25,26}.

In vitro addition of ^{64}Cu (non-carrier free) to hemolysates and subsequent DEAE-cellulose fractionation indicated that $\sim 40\%$ of the added copper was bound to the erythrocuprein fractions and 60%

to the hemoglobin fraction. Further DEAE-cullulose separation of this second fraction indicated that the copper activity was not associated with hemoglobin at pH = 6.2, but occurred in the carbonic anhydrase fraction²⁴.

Furthermore, only ~ 20% of the copper in purified erythrocuprein could be complexed by cyanide ion over an extended period of time²⁴. While this result is difficult to comprehend, since erythrocuprein is stated to have 2 copper atoms/molecule and one would therefore expect complexing of only 0, 50 or 100% of the protein-bound copper, it does indicate that erythrocuprein copper is firmly bound.

From the data of Neumann and Silverberg²⁷ it appears that of the 1 mc ⁶⁴Cu (\equiv 0.5 mgm Cu) injected into a normal human patient, all diffused into erythrocytes within 10 minutes. After 20 hours the red cell ⁶⁴Cu level had dropped by only 50%, but over the same period the dialysable ⁶⁴Cu levels dropped by a factor of 3. Consequently during this time there was considerable stabilisation of the ⁶⁴Cu into a non-dialysable, presumably protein-bound form. The authors further state "Although there is no evidence that erythrocuprein exists as an apoprotein or one with only a partial complement of copper, this must be seriously considered, unless one postulates the existence of a new stable copper protein complex." While the fixation of ⁶⁴Cu might be due to exchange with inactive copper already present in erythrocuprein, this seems unlikely in view of the stability of the copper binding with respect to cyanide.

As a result of the data presented here together with that already in the literature, perhaps the most reasonable hypothesis is the following:

Erythrocuprein is the stable erythrocyte copper protein, containing 2 atoms copper/molecule, and with a molecular weight of 33,000. This protein is able to bind the remainder of the erythrocyte copper, possibly as the amino acid-copper complex shown by Neumann and Silverberg to be the main route for copper to pass from plasma to red cell. During the preparation of erythrocuprein by precipitation with anti-serum this less strongly bound copper is split off and only that which is an integral part of the protein molecule remains. Excess copper, above that bound in these primary or secondary modes, is complexed by yet another erythrocyte protein, possibly carbonic anhydrase.

This system does not account for the dialysable nature of the labile pool, but gel chromatography of hemolysate incubated with ^{64}Cu shows no evidence of small molecular size copper species. Hence unless one postulates that dialysis against a saline-borate buffer ($\text{pH} = 7.4$)²⁶ causes the breakdown of a hemolysate macromolecule which is unaffected by gel chromatography in an acetate buffer ($\text{pH} = 7.1$) these results must remain irreconcilable.

Furthermore, it must be emphasized that the data given herein are the results of only one series of runs, and consequently more work would be required to completely establish their validity. This study was undertaken primarily to complete the description of the overall blood-copper picture, but as seems almost inevitable in research into blood metalloproteins, the situation appears much more complex than was originally anticipated.

CONCLUSIONS

The feasibility studies with ^{64}Cu and ^{131}I showed that the fractionation of plasma samples by gel chromatography provided an excellent gentle method of separating the plasma proteins in such a way that individual proteins containing "inorganic" trace elements could be readily detected. Furthermore, since proteins are eluted in order of molecular size, the detection of a protein-bound trace element at any given elution volume immediately gives some information about one of the most meaningful physical properties of the macromolecule - its size.

When the technique was extended to the detection of protein-bound trace elements in serum by analysis of the protein eluate fractions a further advantage became obvious. Addition of radioisotopes to blood, even in vivo, and subsequent fractionation of the blood does not necessarily reveal all the metalloproteins or metal-protein complexes existing in vivo. This became quite clear from a comparison of the chromatograms of serum labelled in vivo with ^{64}Cu , and serum analysed for copper after fractionation. In the latter case three serum copper fractions were in evidence whereas only ceruloplasmin was seen in the tracer experiment.

The success of the activation analysis experiments for copper and the associated results obtained for iron in serum showed the real potential of the method for the systematic detection of metalloproteins

in biological material. While contamination of the sample prior to, or during, fractionation may produce results which do not reflect the true in vivo situation, these will usually be detected by carrying out in vitro runs involving the addition of the element in question to the sample prior to fractionation. The method is obviously best suited to the identification of protein-bound metals in liquid biological materials such as serum or cerebrospinal fluid, but could be applied to homogenates of solid tissues or extracts therefrom. However, the preparation of the sample required in these instances would greatly increase the danger of contamination.

The other requirement for successful application of the technique is the ability to analyse the element of interest at the low concentrations at which these inorganic elements generally occur in tissue. In most cases the best analytical methods are either neutron activation analysis or atomic absorption spectroscopy. At present the latter technique is preferable to activation analysis in only a few cases, but with the present rapid improvement in instrumentation for atomic absorption, this could well become the method of choice in the near future. Activation analysis suffers from the disadvantage that certain elements cannot be determined due to such unfavourable factors as very long or very short half-lives, low cross-sections, or low isotopic abundances. Furthermore the technique of activation analysis at trace levels is only available to those with high flux irradiation facilities and relatively sophisticated counting equipment.

Most analytical techniques other than the two mentioned above require some pre-treatment or concentration of the sample, and any

such processes increase the risk of contamination dramatically. To be useful for the methods described in this thesis one might arbitrarily require that the limit of detection for the analytical technique used be at least a factor of 100 below the total amount of the element under study.

If one fraction is contaminated in some way during an experiment involving typically 20 or more fractions, this usually becomes quite obvious in analysing the results since a single peak rising above a uniform background is most unlikely to represent a genuine protein-bound metal fraction. Generally at least two adjacent fractions above the background are required to define a peak. In a sense the background is analogous to the noise level in an instrumental analysis and the most desirable situation in the techniques described herein is to have a constant noise level with the highest possible signal to noise ratio.

The in vivo use of radioisotopes to label trace element-binding proteins may also be of considerable use as was illustrated in the manganese experiments. However, in this case, in addition to the possibly deleterious effects of loading the system with an abnormal quantity of the element of interest, there is no way of ascertaining whether all proteins binding the element are in fact labelled at the time of sampling, i.e., not all trace-element protein complexes may be detected. Furthermore, there is virtually no possibility of obtaining quantitative results in this way.

The more specific conclusions to be derived from these experiments are detailed below.

(1) Copper in Serum

Protein-bound copper in serum occurs mainly as ceruloplasmin together with a small quantity bound in the albumin region of the gel chromatograms. A high molecular weight protein which complexes copper at physiological pH has been detected. This protein is eluted at the exclusion limit of Bio-Gel P-200 and therefore has a molecular size consistent with that of a globular protein of molecular weight greater than 200,000. The activation analysis experiment showed this protein could bind at least 1 μgm copper/ml serum and this copper-binding protein is presumably that observed by Fritze and Gietz³² in their in vitro contamination studies. However, these workers showed a very much lower copper-binding capacity for their high molecular weight protein. This difference may be attributed to the binding being in competition with "albumin binding" in the in vitro studies, whereas in the activation analysis experiments the high molecular weight copper binding protein scavenged the gel column ahead of the albumin, and this competition did not arise. That this protein may not be specifically copper-binding is indicated by the work of Hancock who found that very small quantities of ⁶⁵Zn were bound in vitro by a similar sized protein⁴³.

Whether the protein binding is specific or not, it is possible that this protein may be a fourth component in the equilibrium proposed by Neumann and Sass-Kortsak²² involving albumin-bound copper, amino acid-bound copper and ionic copper. Should this be so the quantity of copper ordinarily complexed by this protein in vivo would be minute (<<1% of the total serum copper content).

(ii) Copper in Erythrocytes

Gel chromatography of a human hemolysate, followed by neutron activation analysis for copper showed essentially a single copper component, occurring in an elution position corresponding to a globular protein of molecular weight $\sim 40,000$. This component is obviously the erythrocuprein content of the hemolysate. A minor component of apparent molecular weight $\sim 4,000$, and corresponding to $\sim 3\%$ of the total erythrocyte copper was found. This component could not be attributed to any previously recognized copper protein, and despite its low concentration, would therefore merit further study. It could possibly represent a copper-peptide complex which might in some way be involved in copper transport into the erythrocyte. No ionic copper was detected.

In vitro experiments with carrier-free ^{64}Cu showed that in the absence of EDTA, two hemolysate proteins bind the added copper, leaving no detectable ionic ^{64}Cu , whereas in the presence of the complexing agent only 30% of the radiocopper was bound, by a protein of similar molecular size to erythrocuprein. The remaining 70% was eluted in a volume corresponding to ionic copper.

These results are not apparently reconcilable with previous published work on erythrocyte copper and though a possible explanation of the discrepancies was proposed in the previous chapter (p. 69) more work is needed in this area.

An experiment which might prove valuable here would be the attempted synthesis in vivo of ^{67}Cu -labelled erythrocuprein, followed by in vivo and in vitro studies similar to those described herein,

using ^{64}Cu in an attempt to detect exchange between erythrocyte copper and added ionic copper.

(iii) Manganese in Serum

Work published by others³⁰ on in vitro and short-term in vivo interactions between manganese (II) and serum proteins showed that in such situations the main manganese-binding protein was a β_1 -globulin. This work was extended to gel chromatographic studies which showed this compound to have a molecular weight similar to that of serum albumin, i.e., $\sim 70,000$. This metal-protein complex was found to be relatively labile, and the bound manganese could be readily exchanged with other environmental manganese. These results are in accord with those obtained by previous workers^{30,31}.

A previously undetected manganese protein of molecular weight 200,000 was found. This protein showed no capacity for binding manganese in vitro, or in the short term, in vivo. This compound may be regarded as the true manganoprotein in serum and is apparently not that designated by Cotzias and Bertinchamps²⁹ as transmanganin. This latter was reported as a β_1 -globulin which binds added ^{54}Mn both in vivo and in vitro. Hence it appears that transmanganin is the lower molecular weight manganese-binding protein (molecular weight $\sim 70,000$), though this was not claimed in the most recent publication³⁰ on this subject. The $\sim 200,000$ molecular weight manganese protein which presumably acquires its manganese complement during synthesis (in a similar mechanism to that for copper in ceruloplasmin) could be perhaps best designated as plasmanganin.

Even 30 days after the injection of carrier-free ^{54}Mn both

the high molecular weight protein and the β_1 -globulin binding manganese could be detected in serum. The possibility exists that these are in some form of equilibrium, but this cannot be ascertained by the methods described herein. However, the isolation of the higher molecular weight component would provide a most challenging assignment. Even if all the ~ 0.5 ngm Mn/ml serum was bound in this protein (and this does not appear too likely) and if in addition the protein contained only 1 atom manganese/molecule (again, based upon the composition of known metallo proteins of this molecular size, this is also improbable) the total quantity of the protein in serum could not be more than 2 μ gm/ml.

The in vitro manganese-binding protein would appear by gel chromatographic evidence alone to be albumin, though the electrophoretic work³⁰ shows the metal-binding protein to be a β_1 -globulin. Fritze and Gietz³² found several metals to be bound in the albumin region of gel chromatograms during in vitro work with serum, though a definite displacement was observed in this region in the radioactivity peaks when mixtures of radioelements were added to serum. Together with the results reported here, such data suggests that the generally-accepted concept of relatively non-specific albumin binding of metal ions should perhaps be re-examined to determine whether binding is in fact by albumin or by some other trace protein component.

(iv) Manganese in Erythrocytes

The occurrence of a manganese porphyrin in erythrocytes, as first suggested by Cotzias and Borg³⁴ has been virtually established. Following the appearance of ⁵⁴Mn in erythrocytes after injection of

the isotope into a rabbit, it was shown that the radioisotope was eluted with hemoglobin during gel chromatography of the hemolysate and that following separation of the hemin from the apoprotein globin, the radiomanganese was eluted from a gel chromatographic column using an organic solvent in a volume corresponding to a typical porphyrin molecular weight, i.e., 500-600. There was a suggestion that this manganese porphyrin has a molecular size slightly less than hemin.

In alkaline solution the mangano-porphyrin polymerised in a similar fashion to the hemin. This reaction has long been known for iron porphyrins, both ferrous and ferric, but has not previously been observed for manganese porphyrins⁴⁸.

The existence of such a compound in the erythrocyte might imply that the manganese porphyrin plays some part in the overall respiratory process since one of the symptoms of manganese deficiency in mammals is a breakdown in hemoglobin regeneration. These results have a special significance in view of two recent papers. Fabry et al.⁵³ have found specific binding between apohemoglobin and a manganese meso-porphyrin IX complex together with partial restoration of the globin helical content. Yonetani and Asakura⁵⁴ have demonstrated that a synthesised cytochrome c peroxidase (from apoenzyme and manganese proto-porphyrin IX) has some of the enzymic activity of the naturally occurring heme enzyme. This confirms an early hypothesis⁵⁵ that iron can be replaced to a limited extent by other metals in the prosthetic group of heme-containing enzymes. In view of the extremely low manganese concentration in erythrocytes the question of whether the manganese porphyrin is merely present as "accidental" substitution of iron by manganese, or whether it plays an essential role in the porphyrin metabolism of cells should be of considerable interest to biochemists.

APPENDIX 1

EXAMPLES OF ACTIVATION ANALYSIS DATA

Examples of data treatment for typical neutron activation analysis experiments:

(a) Analysis for protein-bound copper in serum, after fractionation on Bio-Gel P-150 (Fig. IV). In this experiment the samples were irradiated to saturation for ^{64}Cu build-up, and after a 12 hour cooling period leached from the irradiation capsules and counted directly, using a Ge(Li) detector.

A portion of the typical γ -spectrum so obtained is shown in Fig. A1. Only 360 channels of the 1024 recorded are shown, and in the interest of clarity only every second data point (except in activity peaks) has been plotted. A fraction containing only a small quantity of copper has been selected as the example, to better illustrate the influence of other isotopes on the determination of ^{64}Cu . In fractions containing large amounts of copper as in, say, the ceruloplasmin peak the 0.511 MeV peak becomes the dominant feature of the spectrum.

In the particular example shown all but two of the spectral lines are due to ^{82}Br ($t_{1/2} = 35.9$ hr). All fractions in the protein elution region of the gel chromatogram contained this activity, which by its distribution appeared to be protein-bound, but not specifically so in any particular protein. This problem is being

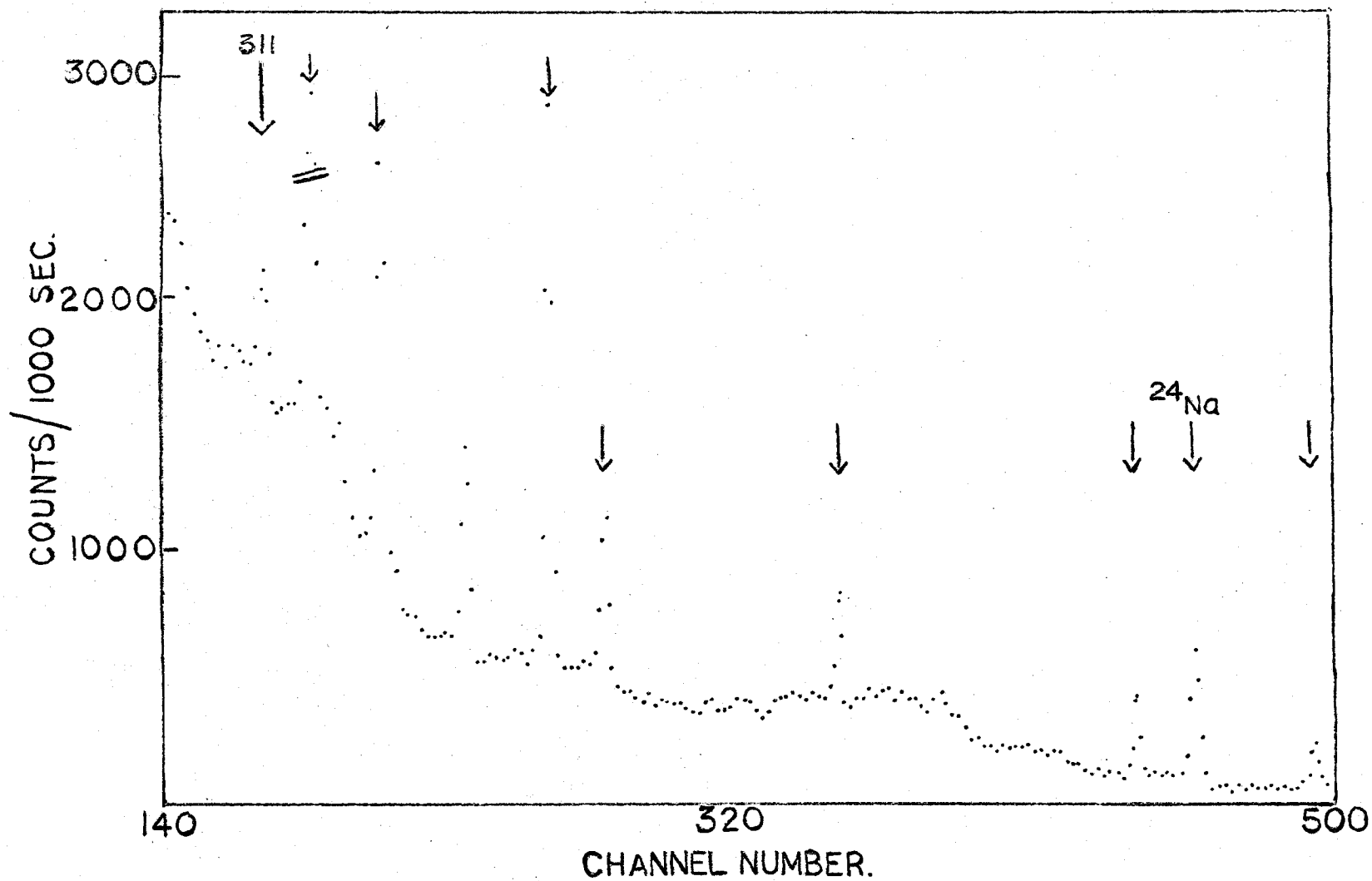


FIG A1

Part of a typical γ -ray spectrum as determined by Ge(Li) counting. Unmarked arrows indicate peaks due to ^{82}Br .

investigated by Firnau and Fritze⁵⁶.

This type of data was handled as follows to obtain the copper content of the fraction:

(i) The counts in the 0.511 MeV peak (top 5 channels) were summed, and the "spectral" background (the sum of the counts in the 5 channels immediately following the peak) was subtracted. This gave a value a counts/1000 sec.

(ii) The value a must be corrected for ²⁴Na contribution to the 0.511 MeV activity. This was done by subtracting from a the quantity b equal to the sum of the counts in the 1.368 MeV ²⁴Na photopeak multiplied by a factor relating the number of counts in the photopeak to those in the 0.511 MeV annihilation radiation peak. This factor as measured in a radiochemically pure solution of ²⁴Na in the same volume and geometry as the experimental samples. For the particular run under discussion the factor was 0.094.

This gave the ²⁴Na-corrected 0.511 MeV peak count rate c, assumed to be entirely due to ⁶⁴Cu activity. Since each sample was counted ~12 hours after the end of irradiation, and the ⁶⁴Cu and ²⁴Na half-lives are relatively similar, no corrections for different rates of decay in the two isotopes were made.

(iii) The ⁶⁴Cu activity c counted at time t was corrected to zero time (end of irradiation) by division by a decay factor d =

$$e^{\frac{-0.693t}{t_{1/2}}}$$

(iv) The Alcan alloy 424 (copper content = 3.7%) samples which served as standards were weighed and dissolved in a volume of dil. HCl equal to that of the protein samples. When their activity

was of a similar order to that of the samples they were counted (this precaution avoided errors due to analyser dead time) and the activity/ngm Cu at $t = 0$ (e) was calculated, again using another decay factor.

(v) The copper content of each fraction was then calculated using the formula:

$$\text{Cu content} = \frac{c}{d} \cdot \frac{1}{e} \text{ ngm.}$$

A typical set of such data is tabulated in Table A1. The errors in such determinations were calculated by standard statistical methods⁵⁷. As would be expected from the examples in Table A1 the percentage error in such fractions as #1 and #26-30 was relatively large (20-30% of the copper content) but dropped to $\sim 5\%$ in the peak copper fractions (#9-14).

(b) When chemical separation and NaI(Tl) counting was carried out, the sample was generally radiochemically pure at the time of counting, so no corrections were needed, either for interfering radioisotopes (as was ²⁴Na above) or for "spectral" background. However, it was necessary to determine the chemical yield of the carrier for the separation, and it was necessary to include this in the data.

An example of the data from such an experiment is given in Table A2. This refers to the data of Fig. I (the determination of protein-bound manganese in rabbit serum). Two samples were irradiated together with one standard (aluminum foil containing 70 ppm manganese).

Table A1

Fraction Number	0.511 MeV c/1000 sec above bg. a	1.368 MeV c/1000 sec in peak	²⁴ Na correction to 0.511 MeV peak c/1000 sec b	⁶⁴ Cu c/1000 sec c	Decay correction d	Spec. act. of standard e	Cu content ngm
1	420	1880	170	250	0.54	142	3.3
2	1280	9600	900	380	0.52	100	7.3
3	1190	2020	190	1000	0.53	139	14
4	1780	2290	210	1570	0.52	109	28
5	1850	2990	280	1570	0.50	144	22
6	2410	4490	420	1990	0.51	114	34
7	4500	1930	180	4320	0.51	144	59
8	6790	1230	110	6680	0.50	111	121
9	17760	3210	300	17460	0.48	135	270
10	17000	930	90	16900	0.47	100	358
11	24600	1480	140	24500	0.58	128	330
12	16320	2280	210	16100	0.58	124	223
13	15010	11090	1000	14000	0.57	121	202
14	9710	6320	590	9120	0.57	118	136
15	9140	21240	2090	7050	0.55	121	106
16	8280	24300	2200	6080	0.44	124	111
17	3650	1430	130	3520	0.54	110	59
18	3020	3240	300	2720	0.54	114	46
19	1780	3190	490	1480	0.52	101	28
20	2170	5220	310	1680	0.52	102	31

Table A1 (Continued)

21	1340/738 sec	3380	740	1030	0.51	127	22
22	2700	7860	620	1960	0.61	123	26
23	1470/831 sec	6640	880	850	0.51	114	18
24	2980	9480	660	2100	0.60	127	28
25	1290	2000	890	630	0.48	122	11
26	1440	9530	430	550	0.58	127	7.5
27	760	4580	430	330	0.47	110	6.4
28	1070	6860	630	440	0.57	134	5.8
29	240	1310	120	120	0.45	98	2.7
30	390	1610	150	240	0.51	132	3.5

Table A2

Fraction	Sample		Standard			Mn content ngm/ fraction
	⁵⁶ Mn act. c/min at t = 0	Chem. yield	⁵⁶ Mn act. c/min at t = 0	Chem. yield	corrected spec. act. at t = 0 ⁵⁶ Mn c/min/ngm Mn	
1	198	81.4%				0.83
2	187	89.4%	49405	52.3%	293	0.72
3	265	89.3%				0.78
4	352	81.3%	67080	71.6%	381	1.13
5	213	91.8%				0.69
6	389	85.3%	161810	93.2%	336	1.35
7	192	89.3%	159840	98.0%	307	0.70
1/2 ml unfractionated serum	682	88.4%	159840	98.0%	307	2.51

APPENDIX 2

EXPERIMENTAL PARAMETERS FOR FIGURES IN CHAPTER 3

N.B. In all experiments, unless otherwise stated, the eluting buffer was 0.15 M ammonium acetate solution (pH 6.8 - 7.2), and the optical density of the column eluate was monitored at 254 m μ .

- Fig. I: 0.4 ml rat serum applied to Bio-Gel P-150 (#37473) column 27 x 1.6 cm. Fraction size 1.5 ml. Rat dosed with ^{64}Cu 24 hours prior to blood sampling.
- Fig. II: as Fig. I except that 1.5 ml rat plasma applied to column.
- Fig. III: 0.5 ml rat plasma applied to Bio-Gel P-150 (#37473) column 31 x 1.6 cm. Fraction size \sim 1.5 ml. Rat dosed with ^{131}I 48 hours prior to blood sampling.
- Fig. IV: 2 ml human serum applied to Bio-Gel P-150 (#44613) column 45 x 2.6 cm. Fraction size 2.5 ml. Copper in eluate determined by neutron activation analysis.
- Fig. V: 2.5 ml human serum applied to Bio-Gel P-150 (#37473) column 44 x 2.6 cm. Fraction size 2.5 ml. Copper in eluate determined by neutron activation analysis and expressed as normalised counts ^{64}Cu per minute.
- Fig. VI: 0.5 ml human serum + ^{64}Cu in 160 μgm Cu(II) solution applied to Bio-Gel P-150 (#37473) column 32 x 1.8 cm. Fraction size 2 ml. Serum + isotope incubated for 30 min prior to application to column.

- Fig. VII: 2 ml human serum applied to Bio-Gel P-150 (#44613) column 45 x 2.6 cm. Fraction size 2.5 ml. Iron in eluate determined by neutron activation analysis, expressed as normalised counts ^{59}Fe per 20 minutes.
- Fig. VIII: 1 ml rabbit serum applied to (a) Bio-Gel P-30, (b) Bio-Gel P-150 (#50243) columns each 18 x 1.6 cm. Fraction sizes ~ 5 ml. Manganese in eluate determined by neutron activation analysis.
- Fig. IX: 5 ml rabbit serum applied to Bio-Gel P-200 (#47493) column 58 x 3 cm. Fraction size ~ 4 ml. Manganese in eluate determined by neutron activation analysis.
- Fig. X: 0.5 ml rabbit serum applied to Bio-Gel P-200 (#47493) column 34 x 1.8 cm. Fraction size 3.5 ml. Manganese in eluate determined by neutron activation analysis.
- Fig. XI: 8 ml rabbit serum applied to Bio-Gel P-200 (#47493) column 58 x 3 cm. Fraction size 6 ml. Sample obtained 12 min. after injection of rabbit with ~ 1 mCi carrier-free ^{54}Mn .
- Fig. XII: 10 ml rabbit serum applied to Bio-Gel P-200 (#47493) column: 58 x 3 cm. Fraction size 10 ml. Sample obtained 5 days after injection of rabbit with ~ 1 mCi carrier-free ^{54}Mn .
- Fig. XIII: 10 ml rabbit plasma applied to Bio-Gel P-200 (#47493) column: 58 x 3 cm. Fraction size 6 ml. Sample obtained 31 days after injection of rabbit with ~ 1 mCi carrier-free ^{54}Mn .

- Fig. XIV: 2 ml rabbit serum + carrier-free ^{54}Mn applied to Bio-Gel P-200 (#47493) column 40 x 2.4 cm. Fraction size \sim 3 ml. Serum + isotope incubated for 30 min. before application to column.
- Fig. XV: 1.5 ml rabbit hemolysate labelled in vivo with ^{54}Mn applied to Bio-Gel P-150 (#50243) column 45 x 1.8 cm. Fraction size 6 ml.
- Fig. XVI: Hemin solution in acetone (ex in vivo ^{54}Mn -labelled erythrocytes) applied to Bio-Bead SX-8 column 65 x 1.8 cm. Eluent acetone. Optical density of eluate monitored at 400 m μ .
- Fig. XVII: Hemin solution in 0.1 M NH_4OH (ex in vivo ^{54}Mn -labelled erythrocytes) applied to Bio-Gel P-4 column 50 x 1.8 cm. Eluent 0.1 M NH_4OH . Eluate optical density monitored at 253 m μ .
- Fig. XVIII: 3 ml human hemolysate applied to Bio-Gel P-60 column 50 x 2.6 cm. Fraction size 4 ml. Copper in eluate determined by neutron activation analysis.
- Fig. XIX: 1 ml human hemolysate + carrier-free ^{64}Cu applied to Bio-Gel P-200 (#47493) column 38 x 2.4 cm. Fraction size 2 ml. Hemolysate + isotope incubated for 4 hr. prior to application.
- Fig. XX: 1 ml human hemolysate + carrier-free ^{64}Cu + 1 mgm EDTA applied to Bio-Gel P-60 column 50 x 2.6 cm. Fraction size 8 ml.

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