MANGANESE IN BLOOD

THE DISTRIBUTION OF MANGANESE IN BLOOD

By

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SCOPE AND CONTENTS:

The distribution of manganese in blood serum and erythrocytes has been investigated using a combination of radioactive tracer method with both gel chromatography and disc gel electrophoresis.

In serum, there are two manganese-binding proteins. The first is aβ_1 globulin with a molecular weight of 70,000. This forms a relatively labile manganese complex both *in vitro* and *in vivo*, and is remarkably similar in both its chromatographic and electrophoretic behaviour to the iron-binding protein, transferrin. The second protein is a higher molecular weight β globulin. It is found to incorporate radiomanganese *in vivo* only, thereupon forming a very stable entity.

In erythrocytes, manganese occurs predominantly in a porphyrin bound to apoglobin, giving rise to a species similar to hemoglobin.

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INTRODUCTION

1.1. General

Even in ancient times the nutritional value of many metallic elements was intuitively recognized. The early Greeks, believing that Mars had imbued iron with strength, used it to counteract physical weakness. And sodium, in the form of salt, has long been a basic dietary supplement for members of the animal kingdom. But an intuitive awareness of the functions of metallic elements could contribute little to any intensive study of the physiological role and fate of such elements. What was needed was the development of analytical methods which could be used to determine at least the presence of these metals. Hence, it was not until the nineteenth century that the biological significance of even a few of the known metals began to be ascertained.

The most biologically abundant metals, such as sodium and calcium, were studied first, with study of the remainder following as the detection limits of available analytical techniques were suitably reduced. For example, colorimetry, which was used in much of the early work, has been replaced where practicable, by techniques such as atomic absorption, neutron activation analysis, and the increased used of radioactive tracers.[1]

Such advancements have opened to investigation the realm of metals existing in minute concentrations,

typically less than one part per million. Such metals are commonly known as trace metals. These can be arbitrarily divided into two classes - the essential trace metals, for which a physiological function is known; and the non-essential trace metals, for which no function has been found.

Even before accurate determinations of the metal concentrations and distributions were possible, the biological roles of many trace elements were elucidated by *in vivo* experiments. Biological disorders induced by metal deficiency and poisoning were often studied in this manner.[2] Similarly, *in vitro* studies have uncovered the role of trace metals as enzymatic cofactors.

Often in biological systems, trace metals are associated with proteins, each of which has a characteristic molecular weight, shape, and charge under physiological conditions. Metal ions can be either tightly bound, thus becoming an integral part of the macromolecule, or loosely bound, forming a weak metal-protein complex. These metal-binding proteins can be fractionated using one or more of their above mentioned properties so that specific metal ion concentrations can be determined in each fraction. Standard methods of protein separation include electrophoresis, gel chromatography, systematic precipitation, ultracentrifugation, and ion exchange.

The trace metal manganese is one such element that

is protein bound. And because of its extremely low concentration in blood, its presence therein was not identified until relatively recently. Most of the early work was concerned with the biological activity of manganese. This was followed by measurements of the total manganese concentration in blood fractions. It was not until 1958 that natural organomanganese compounds were identified in blood.[3]

The aim of the work presented in this thesis is to determine the state of the manganese in both serum and erythrocytes. This has been achieved by the use of a combination of electrophoresis and gel chromatography in conjunction with radioactive manganese-54 tracers, in both *in vitro* and *in vivo* experiments.

1.2. Gel Electrophoresis

Electrophoresis in starch and polyacrylamide gels has become a major analytical tool because of the high resolution attainable with respect to all other electrophoretic methods. The gel matrix, unlike other porous media such as filter paper, is a cross-linked polymer with pores of molecular dimensions, so that a molecular sieving process is combined with electrophoresis. This means that molecules are separated by size as well as by charge differences. Hence, while paper electrophoresis at pH 8.6 gives five major blood serum fractions, starch gel electrophoresis resolves twenty to thirty fractions.[4,5]

Successful use of polyacrylamide gels in electrophoresis was first reported in 1959 by Raymond and Weintraub[6] and Ornstein[7]. This technique has many advantages: the pore size of the gel can be adjusted; starting materials are simple and easily purified chemicals; the gels are physically strong, and are transparent to visible light for the acrylamide concentration range that is most useful.

The high resolution attainable in protein separation by polyacrylamide gel electrophoresis has been ably demonstrated by both Ornstein[8] and Davis[9]. Disc gel electrophoresis in small vertically held tubes, as first described by Davis[9,10], has now become a general separation technique.

Raymond and Nakamichi[11] reported that an increase in polyacrylamide concentration decreases the electrophoretic mobility of proteins. They also showed that for medium gel strengths the pore size is inversely proportional to the square root of the acrylamide concentration.[12]

By varying gel concentrations in electrophoretic runs so that proteins migrate at different rates, and by inserting the resulting data into empirically derived formulae, estimates of protein molecular weights or sizes have been obtained.[11,13,14,15]

For example, Zwaan[15] found that the ratio of the protein mobility in gels of different acrylamide concentration

was directly proportional to the logarithm of the molecular weight. He reasoned that the ratio should be related to the diffusion coefficient or molecular radius rather than the molecular weight. For the five to ten per cent concentration range, he used the formula

$$R_1/R_2 = a \log(\text{Mol. Wt.}) - b$$

where R_i is the protein mobility at gel concentration i, and a and b are constants.

More recently, Hedrick and Smith[13] studied charge and size isomer separation in conjunction with estimates of molecular weights of proteins. They found that for each protein the logarithm of the rate of migration relative to the electrophoretic front was directly proportional to the gel concentration. In such a plot, size isomers gave rise to non-parallel lines intersecting at the zero per cent gel concentration, while charge isomeric proteins gave a series of parallel lines. The slopes of the straight lines obtained as above were ascertained to be directly proportional to the molecular weight. An equation of the following form results:

$$\text{Mol. Wt.} = a \log R_1/R_2 - b$$

where R_i are the relative migration rates at gel concentration i, and a and b are constants.

The pH of the system may be altered to suit the sample being fractionated. Serum proteins[5,6], fruit proteins[17], and ribonucleic acid[18], and acidic or neutral

enzymes[19], have been clearly separated using alkaline media, whereas acidic conditions have been recommended for basic enzymes[20] and histone[21,22] separations.

Although cylindrical gels are most common, use of a square gel has been reported [23], the advantage of this shape being an increased resolving power of the photometric densiometers used to scan the gel.

In preparative gel electrophoresis, efficient sample recovery systems are of vital importance. The sophistication of these techniques has increased remarkably over the past decade.[24,25] Mann and Huang[25] have collated seventeen reports of new and improved systems.

Protein staining techniques involve denaturing the proteins, followed by staining with a suitable dye[9,26] which binds to the protein. The sensitivity of the method depends both on the binding strength of the dye to the sample proteins and the ability to remove excess dye from the system either by electrophoretic destaining[9,27] or repeated washing with a suitable solvent.

1.3. Gel Chromatography

Neutral granulated gels form the stationary phase in column chromatography. Accordingly, separation of macromolecules by this technique is based predominantly on their size differences. For each particular species, an equilibrium is set up between the molecules trapped in the gel matrix and the molecules in the intergel regions.

The pore sizes of the gel regulate the size of the species that can penetrate the gel, so that while small molecules and ions have freedom to move through the whole solvent system, larger molecules have only partial penetration of the gel pores, and very large molecules with no penetration are restricted to the intergel solvent. Each molecular size, therefore, has a limiting pore size below which it is excluded from the intergel regions. This means that when a sample of differently sized molecules is being eluted from a suitable column of granulated gel, the largest molecules will travel fastest, especially if they are totally excluded from the gel pores; the smaller molecules will be retarded by differing amounts, depending on their individual tendencies to diffuse temporarily into the gel phase. Thus the molecules are eluted from the column in order of decreasing molecular size.

Determann[28] has differentiated between gel filtration and gel chromatography. Gel filtration is synonymous with desalting, and applies to the situation in which, in the separation of large and small molecules, the large molecules are completely excluded from the gel, while the small molecules are able to penetrate the gel and are therefore delayed. Gel chromatography describes the case where the molecules of various sizes differ in their relative penetrations of the gel beads, and separation depends on the resolving power of the gel used.

The sieving effects of porous uncharged support matrices, such as charcoal and cellulose, used in adsorption studies, have been reviewed by Deuel.[29] It was also Deuel who first prepared an uncharged gel.[30] Following this breakthrough, column chromatography with starch and agar gels began.[31,32] In 1959, Porath and Flodin[33] reported the discovery of a gel polymer of dextrose, which is now being sold as "Sephadex" and is used extensively in protein separations. Granulated polyacrylamide[34,35] and agarose[36,37] gels have since been developed for chromatographic separations of many hydrophillic molecules such as proteins.

Although there is general agreement among many investigators in this field that the elution volume of a species depends on its molecular size, there is a major variance of opinion as to which size parameter is most significant. One school favours a correlation with molecular radius or diffusion coefficient, while the other prefers the more direct comparison with molecular weight. For proteins with similar frictional ratios, the difference between these two correlations is immaterial. Therefore, the simpler and more helpful relationship between elution volume and molecular weight can often be utilized with a high degree of confidence for a large range of proteins.

The partition coefficient, K_d, which is a measure of the penetration of each particular protein into the gel beads, has been defined by Gelotte[38] by the equation

$$K_d = \frac{V_e - V_o}{V_t - V_o - V_i}$$

where V_e = elution volume of the particular solute

V_o = void volume of the column, and is the elution volume of any totally excluded species

V_t = total column volume

V_i = volume of the gel not accessable to the solvent.

Laurent and Killander[39] proposed the use of the average distribution coefficient, K_{av}, which they define using the equation

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \cdot$$

This average coefficient is related to K_d by the formula

$$K_{av} = \frac{V_t - V_o - V_i}{V_t - V_o} \cdot K_d \cdot$$

Mathematical correlations between elution volumes and Stokes radius, (a), have been made by:

Porath[40] $K_d^{1/3} = k_1 - k_2 a$;

Laurent and Killander[39] $(-\log K_{av})^{\frac{1}{2}} = k_1(k_2 + a)$;

Squire[41] $(V_e/V_o)^{1/3} = k_1 - k_2 a$;

Ackers[42] $a = k_1 + k_2 . \text{erfc}^{-1} . K_d$

where constants k_1 and k_2 are different in each case, and erfc^{-1} is the inverse error function complement.

These relationships have made possible the determination of values for the Stokes radii of many proteins.

Following this theoretical work, equations involving

elution volumes and molecular weights have been derived.[28] Examples are listed:

Porath $K_d^{1/3} = k_1 - k_2 \cdot M^{\frac{1}{2}}$;

Squire $(V_e/V_o) = k_1 - k_2 \cdot M^{1/3}$;

Ackers $K_d = f(M^{1/3}/k_1)$

where M = molecular weight

f = function

and k_i are differing constants as before.

These equations appear to work as accurately as the large number of empirical formulae derived from practical considerations[28,43,44,45].

Andrews[43] has shown that in gel chromatographic protein separations the separation range has three distinct regions. In the major part, there is a near linear relationship between the elution volume and the logarithm of the molecular weight. At both the high and low molecular weight ends the relationship does not hold, and this leads to a negligible separation of molecules in these regions. He has also shown that the linear relationship is independent of pH in the pH range 1.3 to 10.7.

In an attempt to overcome the problem of different molecular frictional ratios, Davison[46] has studied the gel chromatography of proteins, with molecular weights up to one hundred thousand, in denaturing solvents. He found a reasonable correlation of distribution coefficient with log molecular weight. However, the gel resolution was

much worse than that in more common solvent systems, and this limited the number of denatured proteins that could be satisfactorily separated in each run.

In this present work, gel chromatography is used primarily to fractionate the blood proteins in a convenient manner and under relatively mild conditions, in order to retain the physiological equilibria of the various protein-metal complexes, if that is possible. The determination of molecular weights by gel chromatography is still of importance when it comes to identifying particular manganese-binding proteins.

1.4. Trace Elements in Biological Tissues

As analytical techniques have become more sensitive, the interest in the role of inorganic elements in a multitude of biological systems has mushroomed. Classical techniques like colorimetry[1] have been replaced by those new ones which have higher sensitivities for specific elements, such as atomic absorption[47,48,49], and neutron activation[50,51,52,53], polarographic[54] and spectrographic[55,56] analyses.

Multi trace element analyses of single samples are now commonplace, especially in widely studied systems like blood[56,57] and other animal tissues[50,55,58]. A comprehensive compilation of such data involving blood was reported by Bowen in 1963.[1] Almost all the known elements had been studied prior to this time.

The concentration of copper, which is one of the established trace elements, was colorimetrically determined to be approximately one part per million in blood as early as 1929[59], and the plasma and erythrocyte copper concentrations were established at about one part per million each during the forties[60]. Similarly, the zinc concentration was found to be 5.5 parts per million in whole blood as early as 1926[61], with plasma and erythrocyte values established at 2 and 12 parts per million respectively by the fifties[62]. However, determinations of manganese concentrations in blood and blood fractions have not followed this general pathway. The manganese concentrations are much lower than those of copper and zinc, and analytical techniques were just not sensitive enough before the mid-forties for accurate measurement at these low levels. (See Table I, page 13)

The determinations of manganese concentrations listed in Table I utilized neutron activation analysis in conjunction with differing radiochemical separations of the manganese-56 produced, except in the case of Fritze and Robertson[66] who used a purely instrumental approach. The drop in the values obtained may be attributed to a keener awareness of contamination problems, with such an awareness leading to cleaner sampling and handling in the pre-irradiation phase of the determinations.

TABLE I

Determination of manganese concentrations in blood using neutron activation analysis

Year	Concentration of manganese in serum or plasma (ppb.)	Concentration of manganese in blood (ppb.)	Reference
1956	17	24	(63)
1961	8.8	29	(64)
1961	2.6 ± 0.5	11.6 ± 2.5	(51)
1964	2.4 ± 0.1	13.9 ± 0.8	(67)
1966	0.6 ± 0.2	8.5 ± 2.7	(65)
1968	≃ 0.4*	—	(66)

* protein bound

Reports of studies involving the interactions of metal ions with proteins have become more prolific with time, and although much of this work has been performed on *in vitro* systems, the information obtained on binding strengths[68] and specific metal binding sites[69] is undoubtedly also relevant to *in vivo* systems. Techniques such as gel chromatography[68,70], electrophoresis[53,71], dialysis[53,72], ultrafiltration[53], and ion exchange chromatography[56] have been used with much success in the separation and identification of blood fraction proteins which bind metal ions. Gentle separation techniques should be used in order to maintain as closely as possible the physiological equilibria. Since the sample, which was effectively in a closed system in the living state, is transferred during analysis to an open system where contamination by extraneous ions can upset these equilibria, great care is needed to minimize the sources of contamination during sampling and protein separation.

Investigations of the distribution of metal ions among blood proteins have been directed along three lines of attack. The first, and the one most likely to lead to true reflections of the physiological state, involves a study of the distribution of naturally occurring isotopes *in vivo*. The problem is reduced to one of blood sampling and protein fractionation, followed by specific metal analysis of each fraction. The second and third methods

involve the use of radioactive isotopic tracers in both *in vivo* and *in vitro* studies.

Tracer labelling *in vivo* may produce misleading results if the system is not allowed to approach equilibrium because of an overdose of material or of a limiting of the *in vivo* mixing time. The effect of an overdose is much more pronounced where elements are present in very low concentrations, as in the case of the manganese-serum system in which the manganese concentration is about 0.6 ppb. . Because of the low concentration, this system can be expected to be very susceptible to loading with even small quantities of manganese ions. Available serum in an average human is nearly 2.5 litres; in a rabbit, 150 ml.; and in a rat, 10 ml. . Also, 1 mCi of ideally carrier-free manganese-54 has a weight of approximately 120 ng. .[70] Therefore, if all of this manganese is cleared into the animal's blood in one dose, the level of the serum manganese will rise radically, since it is the serum that transports most of the injected manganese ions to the mitochondria-rich organs[3]. (See Table II, page 16)

The significant increase in the manganese concentration, especially in small animals, even for the short time the manganese tracer takes to clear to the organs, could cause irreparable damage to the animal's system, thereby actually altering the system being studied. However, the concentration of manganese in rat liver is

TABLE II

The effect of injections of carrier-free manganese-54 on the manganese levels in sera of animals

Animal	Man	Rabbit	Rat
Total serum volume (ml.)	2,500	150	10
Weight of manganese in this volume (ng.)	1,500	90	6
Weight of manganese after injection of 1 mCi of manganese-54 (ng.)	1,620	210	126
Percentage increase	8	133	2,000

reported to be 1.3 μg./g. of wet perfused tissue.[73] This is equivalent to nearly 18 μg. of manganese in the whole liver. Therefore, once the manganese has reached the liver, the effect of the 1 mCi dose of manganese-54 is reduced to less than 1.7 per cent. The uptake of manganese by other organs would help to lower the effect still further.

Since the overloading effect could be considerable, much care is needed in preparing as true a carrier-free tracer as possible. Thus, very pure reagents are required to obtain the high specific activity necessary for meaningful work.

From an analytical point of view, the use of radioactive tracers is a most convenient approach to the study of metal ion distributions in animal tissues, provided the required precautions are taken. High specific activity tracers make it a very sensitive technique, and the ease in detection of radioactivity upon protein fractionation simplifies the analysis greatly.

The third method, involving the use of radioactive tracers *in vitro*, is open to much more criticism. This is because the *in vitro* conditions may be so far removed from the actual physiological state that meaningless, though consistent results are produced. With this in mind, one can term the method a negative approach, since if a reaction does not work *in vitro* but does *in vivo*, then it can be claimed that the reaction is metabolically important. But

if the reaction proceeds both *in vivo* and *in vitro*, then one can only say that the reaction may be metabolically important. Further independent evidence is necessary for reinforcement of this statement. And if the reaction works *in vitro* but not *in vivo*, then its biological importance is extremely doubtful.

1.5. Metabolic Functions of Manganese

A large number of *in vitro* experiments conducted over the past thirty-five years indicate the participation of manganese in many biological reactions.

Borg and Cotzias[3] found that, in man, injected manganese-56 is cleared rapidly from the blood to the liver and other intracellular sites in the body. Mitochondrial uptake was proposed to explain the retention of manganese in these tissues since it had been previously shown that manganese concentrated in the mitochondria of rat organs.[73,74]

The interdependence of manganese and iron has been studied intensively. Thiers and Vallee[73] found an interdependence in rat liver subcellular fractions. Diez-Ewald *et al*[75] determined that rats fed high doses of manganese had reduced liver iron stores and an increased gastrointestinal iron absorption. With rats on high or low iron diets, however, the total body manganese absorption followed the iron concentration, with much more manganese being excreted in the low than in the high iron cases.

The high specificity of the manganese metabolism was illustrated by Cotzias and Greenough when they showed that injections of iron (II) and iron (III), unlike manganese ($\overline{0}$ to VII), failed to cause deviations from the normal partition of manganese-54 in the body.[76]

Manganese has been found to activate enzymes including arginase[77], cysteine desulphhydrase[78], prolidase[79], and many others. It is involved in oxidative phosphorylation[80,81], and fatty acid[82] and cholesterol[83] sythesis.

Manganese poisoning has been observed in miners who chronically inhale manganese dusts.[84] It is characterized by chronic pneumonopathy and a progressive nervous system disorder similar to Parkinsonism.[85] Excessive doses of manganese salts applied experimentally did not produce this effect; however, liver manganese concentrations were elevated.[86]

An upset in the calcium and phosphorus metabolism and severe rickets were observed by Chornock et al.[87] Although hepatic cirrhosis was observed by Findlay[88] and Hurst[89], Diez-Ewald et al[75] did not observe any, despite large oral doses of manganese given for a month. And mutagenic effects of manganese (II) on the yeast Saccharomyces has been reported by Sarachek.[90]

In both mammals and birds, manganese deficiency is responsible for some abnormalities[91], defective growth[92],

central nervous system problems[93], reproductive dysfunctions[92], and problems with fat metabolism[94].

1.6.1. The Distribution of Manganese in Serum/Plasma

Prior to the sixties, very little work had been done on ascertaining the forms in which manganese existed in blood serum or plasma. Early publications from the laboratory of Cotzias indicated that, in spite of the fact that the body manganese existed in seemingly readily dissociable complexes, the manganese metabolism was highly specific and led mainly through the mitochondria.[3,76]

Cotzias and Bertinchamps[95] recorded the first characterization of a serum protein complex of manganese in 1960. They found that both manganese-54 and iron-59 were concentrated in the β_1 globulin region of serum electrophorogrammes. Use of ultrafiltration showed that both the iron and manganese exchanged with their respective inactive isotopes, but they would not exchange with each other. This, together with the ability of iron, and the inability of manganese, to combine with a purified apotransferrin preparation led to the proposal of the existence of a specific manganese-binding β_1 globulin which they named transmanganin in analogy to transferrin.

Foradori *et al*[53] combined ultrafiltration, isotopic exchange, dialysis, ultracentrifugation, and paper electrophoresis in an *in vitro* study of the bovine and human serum - manganese-54 system. In all cases, they

found that the manganese was closely associated with the serum proteins, although it did exchange with inactive manganese. The electrophoretic studies showed a concentration of manganese in the β globulin region, and separation of the β_1 and β_2 globulins using calcium lactate[96] resulted in the radioactivity migrating with the β_1 globulins. Electrophoresis of manganese-54 in the absence of serum gave no indication of manganese dioxide precipitation as predicted earlier[97], in spite of the alkaline pH and the presence of oxygen in the system. Plasma obtained from rats previously injected with radio-manganese produced electrophoretic patterns identical to those obtained in vitro. They were unable to detect any exchange between the bound manganese and magnesium, thereby corroborating the earlier work of Cotzias and Bertinchamps[95].

Cotzias and Papavasiliou studied the distribution of the naturally occurring manganese-55 in serum. Protein separation was effected by ultracentrifugation, and manganese concentrations were determined by neutron activation analysis.[51] By this means they were able to study the manganese distribution under conditions closer to the ideal physiological state than could be reasonably assumed when radioisotopes are used. A large amount of the manganese was found to be associated with the protein fractions. Acidification to pH 4.4 removed eighty-five to one hundred per cent of the bound manganese from the

proteins, thus illustrating the pH dependence on the manganese-protein complex formation.

Himmelhoch et al[56] fractionated human serum by gradient chromatography on DEAE cellulose columns. Using spark emission spectroscopy, they found a total of 40 ppb. of manganese which was concentrated in a single fraction. They concluded that there was a possibility that the manganese was associated with a specific protein in human serum. The high value obtained for the manganese concentration (see Table I, page 13) was most probably caused by contamination of the system, so that their evidence supporting the concept of a manganese-binding protein in serum is insubstantial.

In order to gather further evidence concerning Cotzias's proposal for the existence of the separate transmanganin species, Panić[71] made an in vitro study of the manganese-serum system, using cattle sera exhibiting different transferrin phenotypes. Following starch gel electrophoresis and subsequent dialysis, the radiomanganese was found to be confined solely in the region of the electrophoretically different transferrin bands. On this basis he proposed that the manganese was associated with the transferrin, and not with some electrophoretically similar β_1 globulin; that is, the so called transmanganin.

In 1965 Holeyšovská[68] reported that there was a very weak interaction between manganese (II) and human

serum albumin. She showed that if a Sephadex G-25 column was saturated with human albumin, and the mole ratio of albumin to manganese (II) was increased, the elution volume of the manganese (II) tended towards that of the albumin.

Indications of the combination of manganese and serum albumin have been found in electrophoretic studies[53,95], but Panić[71] showed that after dialysis there was no radioactivity apparent in the albumin region of his electrophorogrammes, confirming that any complex formation would have to be weak.

The oxidation state of manganese in the β_1 globulin complex was proposed by Cotzias and Bertinchamps[95] to be between +2 and +4. Since no further evidence was presented, this conclusion arose presumably from the fact that magnesium (II) did not exchange with the manganese in the protein complex.

Recent magnetic susceptibility measurements[99] indicate that manganese is indeed bound in the trivalent state in a synthetically prepared manganese-transferrin complex. Electron paramagnetic resonance studies of this complex at room temperature and also at 77°K gave a small manganese (II) signal identical to that from an aqueous manganese (II) solution, but the signal intensity corresponded to less than one per cent of the total manganese present. At 4.2°K no signal was obtained.

Apotransferrin is known to form stable complexes

with several transition metal ions other than iron. Those containing chromium (III), cobalt (III), manganese (III), and copper (II) have been synthesized.[100,101]

It has now been established that each apotransferrin molecule possesses two specific metal binding sites[102], and that the binding sites in the protein are equivalent but independent except in the case of chromium (III)[99] and, under certain conditions, iron (III)[103].

Each binding site appears to involve four or five ligands. pH[103] and spectrophotometric titrations[104] have shown that tyrosyl residues are involved in metal binding; and more recently, difference absorption spectra indicate that tryptophan residues also bind copper (II) and iron (III)[69].

In the first reported study of the long term mixing of manganese and blood in vivo, Evans[70] injected a large dose of manganese-54 into a rabbit and took blood samples at twelve minutes, and five, seven, fourteen, twenty-eight, and ninety-three days. Plasma samples were fractionated on Bio-Gel P-150 columns. The twelve minute sample run contained one radiomanganese-containing protein region in the vicinity of the albumin band. In the other samplings, a second higher molecular weight manganese-binding protein was found as well. Evans estimated the molecular weight of this protein to be of the order of 200,000. This was the first indication of a second manganoprotein in serum.

1.6.2. The Distribution of Manganese in Erythrocytes

Although a manganese porphyrin was first synthesized in 1905 by Zaleski[105], it was not until 1958 that Borg and Cotzias[106] proposed the existence of a naturally occurring manganese porphyrin in human erythrocytes. Their evidence to support this proposal came from a combination of *in vitro* and *in vivo* experiments with manganese-54 and blood. *In vitro* mixing of blood with radiomanganese gave rise to some pick-up of the manganese by the erythrocytes. Repeated saline washes were able to remove most of this. *In vivo* incorporation of manganese by erythrocytes produced a firmly bound manganese component with an insignificant quantity of radioactivity in the stroma. The binding strength of this complex was displayed by its resistance to dialysis, even in the presence of ethylenediaminetetra-acetic acid or relatively high concentrations of inactive manganese. They found that the rate of reappearance of manganese-54 in blood was similar to that of iron-59[107,108], and more closely resembled the production rate of protoporphyrin IX which was labelled with nitrogen-15[109] for convenient detection. Furthermore, the erythrocyte radiomanganese was isolated predominantly in the crystallized hemin. This is notably different from the uptake of zinc-65 by erythrocytes since, although the zinc is incorporated at a similar rate to that of the erythrocyte production, it is known to be associated with carbonic

anhydrase[110] and not with hemoglobin.

Norris and Klein[111] showed that duck erythrocytes took up labelled manganese reversibly *in vitro*, but found no evidence for the association of manganese with the porphyrin molecules. The *in vivo* situation was different from that reported by Borg and Cotzias[106] in that only some of the radiomanganese was isolated in the hemin, the rest presumably being in the hemin supernatant. It was proposed that the radiomanganese was incorporated in the erythrocytes during formation and/or maturation of the cells in the bone marrow, and that the non-heme manganese was to have resulted from a weak coupling between the manganese and cells during circulation.

Goldberg[112] found that an *in vitro* incubation of manganese-54 with chicken erythrocytes for short periods of time gave rise to a hemin preparation containing 0.07 to 1.7 per cent of the added tracer. Cyanide was found to inhibit this incorporation.

Mahoney and Sargent[113] determined that the erythrocyte uptake of manganese-54 in man was irreversible, and that the crystallized hemin contained sixty to seventy per cent of the incorporated radioactivity. No mention was made of the chemical yield of the hemin, so that it is impossible to determine if not all of the manganese was combined with the hemin, or if the effect was caused by a low chemical yield of hemin.

Metalloporphyrins of several non-iron transition elements have been prepared since the turn of the century.[105,114,115,117] Of these it has been shown that those with oxidation states of +2 and +3, such as cobalt and manganese, are easily oxidized and reduced.[115,116,117]

Synthetic hemoglobins made from globins and these metalloporphyrins have been reported. Examples include: nickel mesoporphyrin IX with horse apohemoglobin[118]; a copper porphyrin myoglobin product[119], which was immunologically identical with native myoglobin; and human apohemoglobin with copper (I), manganese, and cobalt mesoporphyrin IX[117]. With the last group, specific globin binding and restoration of globin α helical content on binding were demonstrated, using spectrophotometric and circular dichroism techniques. An attempt was made to prepare both mangnaese and cobalt protoporphyrin IX, as precursors for this globin binding experiment, but the homogeneity of the products was uncertain.[117]

Consideration of the ability to synthesize manganese (II and III) mesoporphyrin IX <u>in vitro</u>, as above, in conjunction with the isolation by several workers of radiomanganese crystallized hemin, leads to the conclusion that manganese is present in red cells as a manganese porphyrin. The minute amounts of this species present in the red cells precludes its isolation from the hemin matrix in sufficient quantities to ensure an unequivocal

characterization. However, strong circumstantial evidence for the existence of the manganese analogue of hemin and related compounds may be elucidated by following the behaviour of the small amounts of radiomanganese labelled product in the bulk matrix of hemin after varying chemical changes.

The characteristic properties of hemin, hematin, and their various derivations have been reasonably defined by many researchers[120], and these have been utilized in the comparison of the porphyrin compounds of iron and manganese[70]. Visible spectrophotometry, potentiometry, and diffusion experiments have significantly aided characterization.[120]

When hemin is dissolved in dilute alkaline solutions, hematin is produced. On standing, or application of heat, aggregation occurs. Initial spectrophotometric evidence indicated the formation of dimeric species.[121,122] Haurowitz[123] reported that hematin in 0.1N sodium hydroxide at 15°C exhibited a declining diffusion rate with time. Up to 0.29 days, the average particle weight was 145,000; and after four days, the average was in excess of 4,000,000. He found that cyanide reduced this aggregation considerably: at 0.75 days, the average particle weight was 1,900; and at 5.7 days, it was 2,300.

Gralén[124] determined sedimentation constants for hematin in alkaline solution corresponding to particle weights of 30,000 to 60,000, and Shack and Clark[125]

determined particle weights of 38,000 to 90,000, in the pH range 9.5 to 12.0; while, in the presence of cyanide at pH 11, a weight of 1,500 was found.

In alkaline solution degradation also occurs with the rapid formation of bile pigments.[126,127] This process still occurs, though at a much slower rate, at pHs as low as 7.2 in the presence of pyridine.[128]

Lemberg and Legge[129] proposed that in the dimerization process, the units were bound by interactions of the carboxyl group of the one with the residual charge on the iron (III) ion of the other. This proposal has been extended by Gallagher and Elliot[122] to explain indications of polymerization in the alkaline hematin-pyridine system. Pyridine forms the weak bispyridineferriporphyrin complex, and high pyridine complexes are needed to minimize polymerization. Solutions conatining fifteen to twenty per cent pyridine are commonly used, especially in spectrophotometry.[122,127]

Cyanide ions form the strong biscyanoferriporphyrin complex with hematin and this has been claimed to exist in solution as a monomeric species.[121] This corresponds to a molecular weight of approximately 670, which is at odds with the later work of Haurowitz and Gralén.

EXPERIMENTAL EQUIPMENT AND PROCEDURES

2.1. Materials and Reagents*

For gel chromatography in aqueous solutions porous acrylamide gels were used. These are sold under the trade name Bio-Gel P-X (Bio-Rad Laboratories; Richmond, California, U.S.A.), and are available in a series of experimentally orientated molecular weight fractionation ranges and mesh sizes. The fractionation range of a specific gel is indicated by a number X which denotes that molecules with molecular weights of the order of X,000 are excluded from the gel and are eluted at the void volume of the column. The resolving power of the gel depends on the mesh size of the polyacrylamide beads - the smaller the mesh, the better the resolution. Unfortunately, the flow rate is an inverse function of the mesh size, so that a compromise between resolution and flow rate must be made in practical applications.

Although the gel manufacturing process is standardized, different batches of gel have differing chromatographic properties. Improvements over the past

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

years have increased significantly the flow rates while maintaining the resolving power of the gel. However, the reliability of the quoted fractionation range of each batch, especially for the P-100 to P-300 gels important in protein fractionations, is open to question and should be checked experimentally.

Bio-Gel P-200 (#47493, 15-1-68) was used extensively to fractionate plasma and erythrocyte proteins. Bio-Gel P6 (#54493, 27-3-68) was used in all desalting experiments. For chromatography at pH 7.4, a buffer solution of 0.15M ammonium acetate was prepared by diluting Aristar brand concentrated ammonia and acetic acid (B.D.H.) with deionized distilled water. A prewashed polyethylene container was used to store this high purity solution. A column of Chelex 100 (Bio-Rad Laboratories), in the ammonia form, was incorporated at the base of the buffer reservoir to maintain low metal ion concentrations and reduce the risk of contamination by extraneous metal ions.

The elutant for alkaline chromatography was a solution of 0.01M disodium ethylenediaminetetraacetic acid buffered at pH 9.4 with 0.1M ammonium chloride.

Sephadex LH-20 (Pharmacia, Lot 1152), which is an alkylated form of Sephadex G-25, was the matrix for the chromatography of hemin in organic solution. In this system, the elutant was a mixture of twenty per cent pyridine and two per cent acetylacetone in methanol.

Tracers

Radioactive tracers were usually produced in the McMaster nuclear reactor. Sodium-24, which has a half life of fifteen hours, decays by both beta and gamma emission, with gamma energies of 1.368 and 2.753 Mev., and a beta energy of 1.39 Mev. . Manganese-54 decays by electron capture, emitting a gamma ray of energy 0.835 Mev. with a half life of 300±14 days.

Sodium-24 was prepared for the estimation of the total column volume available to the solute.

Carrier-free manganese-54 was produced by irradiating spectroscopically pure iron, and after a suitable "cooling" period, by chemically separating the manganese and iron by the method of Fritze and Robertson[130] as described below.

The iron pellet was irradiated for five months and allowed to "cool" for four months to reduce the radioactivity from the iron-59 by more than eighty per cent to approximately 2mCi. The iron was leached with acid to remove surface contaminants, and then dissolved in 25 ml. of concentrated redistilled hydrochloric acid. Chlorine gas was bubbled through the heated solution to oxidize all the iron to iron (III).

A small aliquot was titrated with 0.1N sodium hydroxide, using the precipitation of ferric hydroxide as indicator to determine the acidity of the solution at this

juncture. The sample acidity was then adjusted to 8M, and the solution extracted with isopropyl ether preequilibrated with 8M hydrochloric acid. More chlorine was dissolved in the aqueous phase before the second extraction as an added precaution to maintain the remaining iron as iron (III), and the iron (III)-containing isopropyl ether from each extraction was discarded.

The greenish manganese-54 - containing aqueous phase (also containing iron, and possibly cobalt and nickel) was evaporated to dryness in a teflon beaker. The residue was then dissolved in a minimal amount of concentrated hydrochloric acid.

This was applied to a column of Dowex AG 1-X8 (200-400 mesh, 0.3 x 26 cm.) and washed with 12M hydrochloric acid. When traces of manganese-54 started to be eluted , the eluant was changed to 6M hydrochloric acid to concentrate the manganese-54. The sample (1 ml.) was then taken to dryness in a teflon beaker after the addition of one drop of perchloric acid. An almost colourless residue, estimated to be several microgrammes, was obtained. This was dissolved in 1 ml. of 0.01M hydrochloric acid containing approximately 100 ug. of sodium sulphite to give a manganese-54 stock solution. The yield of manganese-54 was approximately 0.75 mCi, and there was no detectable ^{59}Fe or ^{51}Cr, produced by the reaction $^{54}Fe(n,\alpha)^{51}Cr$, in the stock solution.

Disc Gel Electrophoresis

The specific reagents needed for disc gel electrophoresis are listed in Table III, page 35. Stock solutions were prepared from these reagents and distilled water, as recommended by Davis[9] (see Table IV, page 36), and stored at 0°C.

Fixative solution (10 per cent aqueous trichloroacetic acid), staining solution (1 per cent amido black in 7 per cent acetic acid), and destaining solution (7 per cent aqueous acetic acid) were prepared as necessary. Fresh buffer solution was made for each electrophoresis by a tenfold dilution of the stock solution.

2.2. Instrumentation

In gel chromatography on Bio-Gel columns (in both ammonium acetate and ammonium chloride systems), the eluate fractions were collected on an L.K.B. Ultra-Rac fraction collector. The protein, or hematin, content of the eluate was monitored with an L.K.B. Uvicord Spectrophotometer at a wavelength of 254 nm. .

A circular fraction collector (Packard Instrument Company) was used in the hemin chromatography. The porphyrin concentration was determined with a Bausch and Lomb Spectronic 20 spectrophotometer.

The disc gel electrophoresis apparatus was powered by a variable voltage (500 volts maximum), direct current

TABLE III

Reagents for Polyacrylamide Gel Electrophoresis

1. Acrylamide (Eastman Organic Chemicals, 5521).
2. N,N'-Methylenebisacrylamide (BIS), (Eastman, 8383).
3. 2-amino-2-hydroxymethyl-1,3-propandiol, or trishydroxymethylaminomethane (THAM).
4. N,N,N',N'-tetramethylenediamine (TEMED), (Eastman, 8178).
5. Reagent Grade hydrochloric acid.
6. Acetic acid.
7. Glycine.
8. Sucrose.
9. Amido Black 10-B (lot 25583 - B.D.H.).
10. Bromo-phenol Blue.

TABLE IV

Reagents for Stock Solutions

Stock Solution 1	Stock Solution 2	Buffer
48 ml. 1M hydrochloric acid	28 g. acrylamide	6.0 g. THAM
36.6 g. THAM	0.735 g. BIS	28.8 g. glycine
0.23 ml. TEMED	Water to 100 ml.	Water to 1 l. (pH 8.3)
Water to 100 ml. (pH 8.9)		

(150 milliamperes maximum) power supply (Gelman Instrument Company).

Radioactivity measurements were made by gamma ray scintillation spectroscopy with one of the following units:

1. 3x3 inch NaI (Tl) crystal combined with a Victoreen linear amplifier (DD2, Model 851A), and a Nuclear-Chicago scaler-timer.
2. 3x3 inch NaI (Tl) crystal in conjunction with a Victoreen linear amplifier (DD2, Model 851A) and a Nuclear Data analyzer.
3. A coaxial Ge (Li) solid state detector with an Ortec 118A preamplifier, Hewlett-Packard (5582A) linear amplifier, and Nuclear Data (series 2200) 1024 channel analyzer.
4. 3x3 inch well-type NaI (Tl) crystal with a Victoreen linear amplifier (DD2, Model 851A) and a Nuclear-Chicago scaler-timer.

The last assembly was used for low level manganese-54 counting.

Reactor

Neutron irradiations were carried out in the McMaster Reactor (thermal flux $\approx 2 \times 10^{13}$ n.cm.$^{-2}$ .sec^{-1}; cadmium ratio of approximately 20). Short term irradiations (less than twenty minutes) utilized the pneumatic system with a thermal neutron flux greater than 1×10^{13} n.cm.$^{-2}$ .sec^{-1}.

2.3. Disc Gel Electrophoresis

Apparatus

A large sized glass tube (1.6x20 cm.) was used, rather than the smaller tubes generally used, so that samples of up to two millilitres could be applied without significantly altering the resolving power of the technique by overloading the column. The upper (cathodic) reservoir was formed by connecting a widenecked "Nalgene" funnel to the glass cylinder by means of a rubber sleeve, and a polystyrene container served as the lower (anodic) reservoir. A ring clamped above the lower reservoir held the upper reservoir and electrophoresis tube in position.

Cylindrical graphite electrodes made from dry cell batteries were found to be inadequate for the relatively large currents needed for optimum electrophoretic conditions. Electrode digestion caused by rapid hydrogen and oxygen production at the surface was the major drawback. Platinum electrodes were used because of their relative resistance to attack by hydrogen and oxygen and their overall durability. Although the positions of the electrodes in the reservoirs did not visibly affect electrophoretic behaviour of standard samples, care was taken to ensure that the anodic electrodes were set to one side of the gel-containing tube to prevent liberated oxygen from forming an insulating layer between the gel and the

electrolyte. To avoid any side effects, the electrodes were used specifically as either cathodes or anodes.

The high current needed during electrophoresis led to considerable heating of the gel column at room temperature. This effect was minimized by conducting all electrophoresis in a refrigerator at 0°C, at which temperature currents of up to thirty milliamperes could be utilized.

After electrophoresis, gels were cut under water using a razor blade, with the water acting as a lubricant to ensure neat slices.

Electrophoretic Procedures

Stock solutions 1 and 2 were taken from the refrigerator and warmed to room temperature. Meanwhile, the electrophoretic tube was scrubbed out, rinsed with distilled water, and drained. An appropriately sized rubber stopper, wide end inwards, sealed one end of the tube which was positioned vertically by standing the stopper on a horizontal surface, thereby ensuring that the polymerized gel column had a flat bottom. The gel solution (5.5 per cent with respect to acrylamide), prepared by mixing 7.5 ml. of stock solution 1, 15 ml. of stock solution 2, 60 ml. of distilled water, and 0.15 g. of ammonium persulphate, was poured into the vertical tube. Water was carefully layered on top, using a small rubber

bulbed pipette, to produce a flat surface between water and gel.

On completion of polymerization (30-45 minutes), the supernatant water was poured off, and the top of the column washed several times with distilled water. The stopper was withdrawn from the other end, care being taken not to put stress on the gel and thereby separate it from the glass surface.

The electrophoresis apparatus was then assembled as previously described; buffer was added to the cathodic reservoir; and the bottom of the gel was submerged at least one-quarter inch below the surface of the anodic buffer solution. Air bubbles trapped at this interface were removed by rocking the gel tube sideways. Sufficient bromophenol blue solution (10^{-3} per cent in water) was stirred into the upper reservoir buffer to make a pale blue solution.

The sample, in a forty per cent sucrose solution as recommended by Dietz and Lubrano[19], was then layered on top of the gel column, and electrophoresis was started with a current of approximately 15 mamp. . As the sample permeated the gel, the current was found to increase, eventually doubling after one hour as the resistance of the system decreased. After stabilization, the current was maintained at 25-30 mamp. until electrophoresis was completed. With this current, the electrophoretic front,

commonly displayed by the bromo-phenol blue, migrated at a rate of approximately 2.5 cm./hr. .

Following electrophoresis, the detached gel tube was checked for coloured bands, and their migration distances were recorded. The tube was then immersed in a cold water bath, and the gel separated from the walls by gently rimming the inside of the tube with a long flat spatula, with the water acting as a lubricant. When this procedure had been carried out at each end, the loosened gel was slid from the tube using minimum pressure from a close fitting plunger.

In the preparation of gel discs, the flat open end of the gel tube provided the necessary restraint on the gel to enable clean slices to be obtained using a razor blade. The slices were then dried on porous paper and weighed. The widths of the gel blocks were then calculated from these weights.

Next, radioactivity in each slice was measured using a 3x3 inch sodium iodide detector in conjunction with a single channel analyzer. A histogramme of count rate per disc width versus the distance of the mid-point of each disc from the electrophoretic origin gave the distribution of the radiactive isotope down the gel column.

Because of the inherent tendency of the electrophoretic protein bands to diffuse through the gel after electrophoresis, thereby reducing their resolution, protein

fixing had to be carried out as soon as possible. Since the fixing process is also diffusion controlled, the surface area of the gel cylinder was increased sixty-six per cent by cutting the gel lengthwise into two halves with a razor blade, once more using water as a lubricant. Initially, the diameter was 1.6 cm., so that after bisection no point in the gel was more than 0.4 cm. from a surface. Overnight fixing with ten per cent trichloroacetic acid usually lasted between ten and fifteen hours. The gel slices were then removed from the fixing solution, washed with distilled water, and stained with a one per cent solution of Amido Black 10-B in seven per cent acetic acid for a period of six hours. They were then destained by washing in successive seven per cent acetic acid solutions until the background staining was minimized. At this juncture, the relative positions of the stained protein bands were recorded and compared with similar data from the coloured bands compiled just after electrophoresis.

The blue band of the albumin bromo-phenol blue complex was arbitrarily given an R_f value of 1.00 and all other R_f values were determined relative to this band. In the electrophoresis of serum, this band travelled with the albumin fraction, so that the prealbumin bands and the electrophoretic front had R_f values greater than unity.

2.4. Gel Chromatography

Column Preparation

Since this work involved trace amounts of metal ions, it was necessary to take as many precautions as possible to minimize metal ion contamination in the gel matrix and the column containers.

The glass and quartz columns used for tracer experiments were soaked in *aqua regia* for twelve hours to leach out metal ions in the vicinity of the surface, and generally to clean the surface. After thorough washing with deionized water, they were dried and then siliconized with a one per cent solution of dimethyldichlorosilane in benzene (Bio-Rad Laboratories).

Cotton wool plugs to support the bottom of the gel columns were also given the acid treatment, as was white beach sand which was used on occasion to increase the plug size without clogging the column, and to produce faster flow rates and more durable columns.

For the columns at pH 7.4, the dry gels were swollen for suitable periods in the pure 0.15M ammonium acetate buffer containing 0.01M sodium cyanide. Gel columns were then prepared in the usual manner by allowing the gel slurry to settle under gravity, following which the column was more firmly packed under a low pressure head to maintain a slow flow rate. After column stabilization, the pressure head was increased until the desired permanent

flow rate was attained.

P-200 gels were de-aerated under vacuum prior to column formation to ensure that the beads were saturated with buffer and thereby increase their resolving power. The low exclusion limit gels did not require this pre-treatment.

Each column was washed with several column volumes of cyanide-ammonium acetate buffer, and then more thoroughly washed with pure ammonium acetate solution which was added from a glass reservoir at the top of the column via a short column of Chelex 100 cation exchange resin in the ammonia form.

The columns at pH 9.4 were prepared in a similar manner by soaking the appropriate gels in a solution of 0.01M ethylenediaminetetraacetic acid in 0.1M ammonium chloride. Because of the high concentration of ethylenediaminetetraacetic acid in this system, and the high pH, no further precautions were taken to ensure the cleanliness of the system once it had been set up and extensively washed with the clean buffer.

LH-20 columns were packed similarly, with acetylacetone replacing ethylenediaminetetraacetic acid as the scavenger for extraneous metal ions which could possibly contaminate the system.

Columns at pH 7 were stable for long periods (up to a year, at least) even though no special precautions, apart

from the addition of a few crystals of sodium azide to inhibit fungal growth, were taken. On the other hand, P-200 columns at pH 9.4 had a working life of approximately three to four weeks, after which time the swelling of hydrolyzed gel beads both significantly altered the chromatographic pattern and clogged up the columns so as to render them experimentally useless. P-6 columns were more resistant to hydrolysis, and generally lasted up to six months at pH 9.4.

Column Chromatographic Procedures

In the case of the Bio-Gel columns, at both pH 7 and pH 9, two methods of sample application were employed.

Firstly, the buffer above the gel bed was allowed to drain through the column until the flat surface of the gel was dry. The sample was then carefully applied to this surface using a glass pipette, and allowed to soak in. The sample was washed into the gel bed with several discrete applications of small amounts of buffer. After the sample had penetrated a suitable distance, more buffer was added up to a convenient level, the reservoir placed on top of the column, and the pressure head adjusted so that elution proceeded at a reasonable rate.

Secondly, sucrose was dissolved in the sample solution to form a solution somewhat denser than the eluting buffer. This was then gently layered on the flat surface of the column under the buffer already there. Care in this

operation precluded any significant mixing of these two phases. The sample was then allowed to soak in to a safe distance, more buffer was added to the top of the column, the reservoir replaced, and the run continued as before.

In Sephadex LH-20 chromatographic runs, the less elegant first method of sample application was employed throughout.

Eluate fractions in tracer experiments were collected in suitably sized test tubes in preparation for γ ray scintillation counting.

Total elution times depended on the gel type, column size, and flow rate. Typically, Bio-Gel P-6 chromatography took from two to six hours; Bio-Gel P-200, from twenty to forty hours; and Sephadex LH-20, from four to eight hours.

2.5. Sample Preparation

In all *in vitro* radioactive tracer experiments human serum, plasma, and erythrocytes were used, while rat plasma and erythrocytes were employed for *in vivo* studies. Blood samples were withdrawn using clean stainless steel needles and plastic syringes, and were stored in quartz centrifuge tubes.

Serum was prepared from human blood by allowing the blood to coagulate at 37°C for two to four hours and then centrifuging this at low speed. Only non-hemolyzed serum

was used in tracer work.

Citrate was used as an anticoagulant in the preparation of plasma instead of heparin because of the reportedly high concentration of manganese in commercially available heparin,[70,131]

Following the removal of plasma, the remaining erythrocytes were washed at least four times with isotonic sodium chloride solution, separations being effected by centrifugation at each step. The white layer on top of the erythrocytes was removed at the same time. The erythrocytes were hemolyzed by mixing 1 part of packed cells with 1.5 parts of distilled deionized waster and 0.4 parts of toluene. Repeated vigorous shaking extracted the cell stroma from lysed cells into the toluene phase. The two phases were separated by centrifugation. The lower hemolysate layer was pipetted out, filtered through Whatman paper to remove traces of solid and toluene, and stored in a stoppered polythene or quartz container at $0^{o}C$.

Hemin was prepared using a modification by Fritze and Robertson[130] of the method of Lewis[132], since this gave much better yields of hemin than other methods[120]. The modified method is described below.

0.2 ml. of 2M hydrochloric acid was added with stirring to 2 ml. of hemolysate, whereupon the solution turned dark brown as the heme and globin were separated. This solution was added, drop by drop, with stirring,

into 12 ml. of acetone to precipitate out the globin as a grey-white mass. Five minutes standing with occasional stirring completed this process. The globin was then removed by filtration under a slight vacuum through a sintered glass crucible, and washed with no more than 8 ml. of 0.1M hydrochloric acid in acetone in small portions to remove excess hemin. This washing process was determined by colour change, since hemin in acetone is brown and the globin is white. Excess washing presented a problem in that the globin does have a low solubility in the hydrochloric acid - acetone solution, and any globin left in solution will precipitate with the hemin, giving rise to erroneous values for chemical yields. By applying more vacuum after most of the hemin-acetone solution was removed, the excess liquid associated with the precipitate was extracted. Now only a few millilitres of acid-acetone were needed to wash the globin clean.

The filtrate was then transferred to a suitable beaker, and 25 ml. of 0.1M hydrochloric acid was added slowly to start the hemin precipitation. The solution was gently heated (temperature 80°C) to boil off the acetone and coagulate the hemin. This was continued until the brown colour of the solution had faded considerably, and then the crystallized hemin was filtered off and washed with 0.1M hydrochloric acid. This product, dissolved in pyridine with no residue, was shown by Sephadex LH-20 chromatography to be more than ninety-eight per cent pure hemin.

RESULTS AND DISCUSSION

3.1. In Vitro Addition of Manganese-54 to Serum

In this work, fresh human serum was prepared for each new experiment in order to minimize decomposition effects that could lead to erroneous and misleading results. The serum was stored in sealed quartz tubes at 0°C after being spiked with carrier-free manganese, and samples were withdrawn with clean pipettes from the bulk solution as required. Protein fractionation was achieved by disc gel electrophoresis or gel chromatography, and the radiomanganese concentrations were determined by gamma scintillation counting.

Spikes of less than 100 nCi. of manganese-54 per ml. of serum were used. Theoretical calculations (see Section 1.4.) show that this is equivalent to adding less than 12 pg. of manganese, or two per cent of the total manganese in the serum. This would be the minimum quantity of manganese that could be added to the serum system. Additional manganese would be introduced from two major sources. Firstly, the reagents used in the preparation of manganese-54 would contain minute amounts of manganese-55, giving rise to a constant carrier concentration in the manganese-54 solution. The second source would arise from general contamination in the spiking experiment from manganese in

reagents used and surface leaching of manganese from reaction vessels. The concentration of manganese introduced to the system by general contamination would be variable. Therefore, precautions were taken to keep it at a minimum, since addition of excess quantities of manganese could give rise to misleading results. For example, a dust particle containing 1 ng. of manganese, if dissolved in 1 ml. of serum, would raise the manganese concentration by nearly two hundred per cent. Accordingly, in this context, "excessive" quantities are small on an absolute scale.

Gel chromatography of serum on Bio-Gel P-200 resolves the serum proteins into three major molecular size groups, as determined by the absorbance measured at 254 nm., followed by a low molecular size band in which free ions and low molecular weight species are to be found. The first protein band contains high molecular weight species that are excluded from the gel beads (the so called lipoprotein band). The second band is made up of species with molecular weights in the order of 150,000 and includes γ globulins (the γ globulin band). The third protein band contains mainly albumin and lesser amounts of other size isomers (the albumin band).

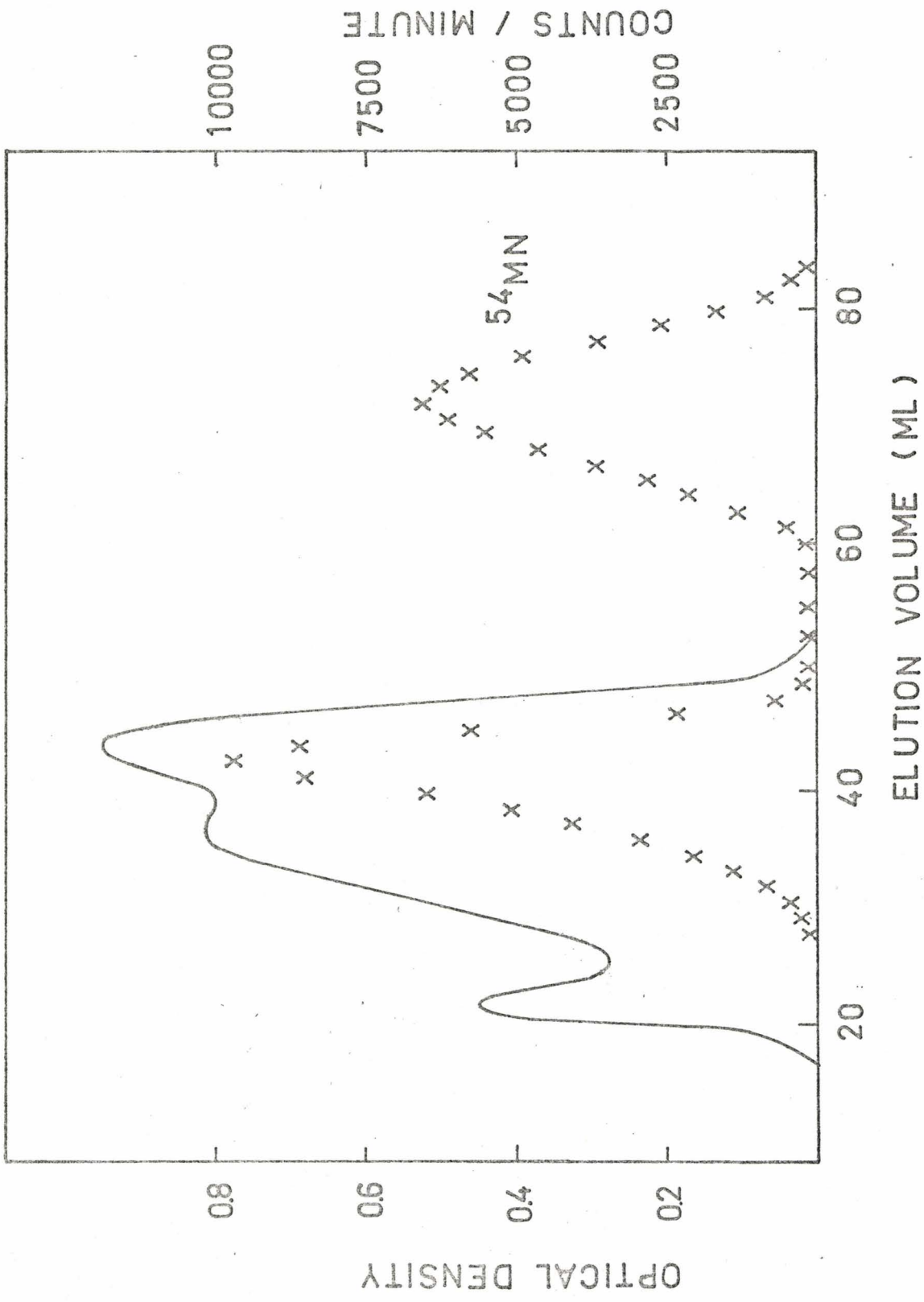
P-200 fractionations of the serum - manganese-54 samples always led to chromatogrammes in which there were two bands of radiomanganese - one in the ionic manganese

region, and the other aligned with the albumin band (see Figure I, page 52). The closeness of the elution positions of the manganese-binding protein (or proteins) and the serum albumin indicates that, if these proteins are similarly shaped, then their molecular weights will be similar. The molecular weight of human albumin is currently given as 68,500[133,134], so the serum protein which binds manganese has an apparent molecular weight in this region. Furthermore, since the shapes of the protein bound and ionic manganese bands obtained on chromatography were identical, and since the ionic peak consists of essentially only one molecular size form of manganese, it follows that only one protein binds manganese. Several manganese proteins may have similar molecular weights, but this is rather unlikely.

Appreciable, though variable, amounts of the tracer were found in the protein region of the chromatogrammes. The major factors controlling the quantity of manganese picked up seem to be sample contamination and the mixing time allowed. Contamination of the system, either by foreign ions or nonradioactive manganese, reduces on a statistical basis the number of radioactive ions which can become attached to the proteins. Hence, the more metal-free the system is, the better the tracer uptake. This point was illustrated by fractionating on P-200 columns four different samples of tracer and serum after only short

FIGURE I

Serum Spiked with Manganese-54 and Chromatographed on a P-200 (#47493) Gel Column (38x1.6 cm.). Sample size, 0.5 ml.


COUNTS / MINUTE
10000
7500
5000
2500
^{54}MN
OPTICAL DENSITY
0.8
0.6
0.4
0.2
20
40
60
80
ELUTION VOLUME (ML)

mixing times. See Table V, page 54. Therefore the use of a clean eluant and gel column is obligatory, and care in the serum preparation is vital as well.

Also, the accumulation of manganese-54 by serum proteins appeared to be time dependent, taking several days to reach a maximum. Results are tabulated in Table VI, page 55.

The variability of the contamination in different experiments negated the establishment of a specific manganese pick-up rate.

Variations in column dimensions and flow rates significantly altered the distribution of manganese-54 between the protein bound and ionic regions of the chromatogrammes. Use of a fast flowing P-6 column (24x1.8 cm., column volume of 61 ml.) which was used for gel filtration gave rise to a higher percentage of manganese in the protein fractions than a longer but similarly sized P-200 column (37x1.6 cm., volume of 74 ml.) with a much slower flow rate. See Table VII, page 56.

This result may be explained by consideration of the rate at which equilibrium is attained in the system. since the initial manganese pick-up was not very rapid, it could be expected that the dissociation of the manganese-protein complex is also a slow process. In the dynamic chromatographic system, the dissociated manganese is retarded and therefore lost to the manganese-protein system,

TABLE V

Effects of random contamination on the manganese-serum system

Mixing Time (hr.)	Percentage of Manganese-54 in Protein Fraction
0.25	3
4.00	11
0.50	18
1.00	23

TABLE VI

Effect of mixing time on the manganese-serum system

Mixing Time	Percentage of Protein Bound Manganese
1 hour	23
3 days	67
6 days	80
11.5 days	85

TABLE VII

Effect of physical properties of columns on the manganese-serum system

Column	Mixing Time (days)	Percentage of Protein Bound Manganese-54
P-200	3	36
P-6	15	97
P-200	23	39

and so produces further dissociation. The manganese-binding proteins are eluted from a P-6 column in less than an hour under normal flow rates, whereas with P-200 chromatography, eight to ten hours are needed. This gives rise to much more dissociation of the complex in the P-200 chromatographic runs.

On the other hand, serum chromatographed on either P-200 or P-6 columns exhibited a radiomanganese-protein complex which was stable in 0.15M ammonium acetate buffer for long periods when stored at 0°C. See Table VIII, page 58. This stability on storage is illustrative of the durability of the complex in the static state, and is independent of happenings in the dynamic state.

Incubation of 0.05M ethylenediaminetetraacetic acid with the manganese-54 serum for one hour removed all but 0.02 per cent of the tracer from the serum protein region following P-200 gel chromatography. Hence, the manganese-ethylenediaminetetraacetic acid complex is more stable than that formed by manganese and the reactive protein.

When citrated plasma was used, manganese-54 was incorporated by the proteins, although much less readily than in the case of serum. See Table IX, page 59. Whether this is an effect of manganese-citrate complex formation or an example of addition of extraneous metal contamination with the reagent is open to question. It has been shown that heparin preparations contain relatively high

TABLE VIII

Stability of manganese-protein complex on storage

Sample	Original Column	Second Fractionation Column	Time between Fractionations (days)	% Protein Bound ^{54}Mn
serum	P-200	P-200	13	91
serum	P-6	P-6	11	98
plasma	P-6	P-6	22	58
serum	P-200	P-6	51	98

TABLE IX

Uptake of manganese-54 by plasma

Mixing Time	Percentage of Protein Bound Manganese-54
25 minutes	1
5 days	8
11 days	34

concentrations of manganese[70,131], and in this case it is the metal contamination effect that alters the system under study.

Disc gel electrophoresis experiments were conducted using a 5.5 per cent acrylamide gel. This gel fractionated the serum proteins much more conveniently than did the seven per cent gel recommended by Davis in that the β globulin band was more clearly separated from the electrophoretically slower proteins. See Figure II, page 61. This phenomenon has been used by Zwaan[15] and others in molecular weight determinations.

After electrophoresis of manganese-54 - spiked serum and plasma samples, the gel was sliced (see Section 2.3.), and the manganese-54 count rate of each slice was determined. In the resulting electrophorogrammes, the tracer was found to be concentrated in the β globulin band, in the post- β globulin region, and in the cathodic solution. See Figure II, page 61, and Table X, page 62. The appearance of a manganese-binding β globulin is in accord with previous reports by Foradori et al[53], and Cotzias and Bertinchamps[95].

The manganese in the cathodic solution can be related to ionic manganese coming from two sources: the manganese not protein bound in the initial sample, and the manganese stripped from the protein-manganese complex during electrophoresis. The presence of the tracer in the

FIGURE II

Electrophoresis of Serum Labelled *In Vitro* with Manganese-54. Sample size, 1 ml.

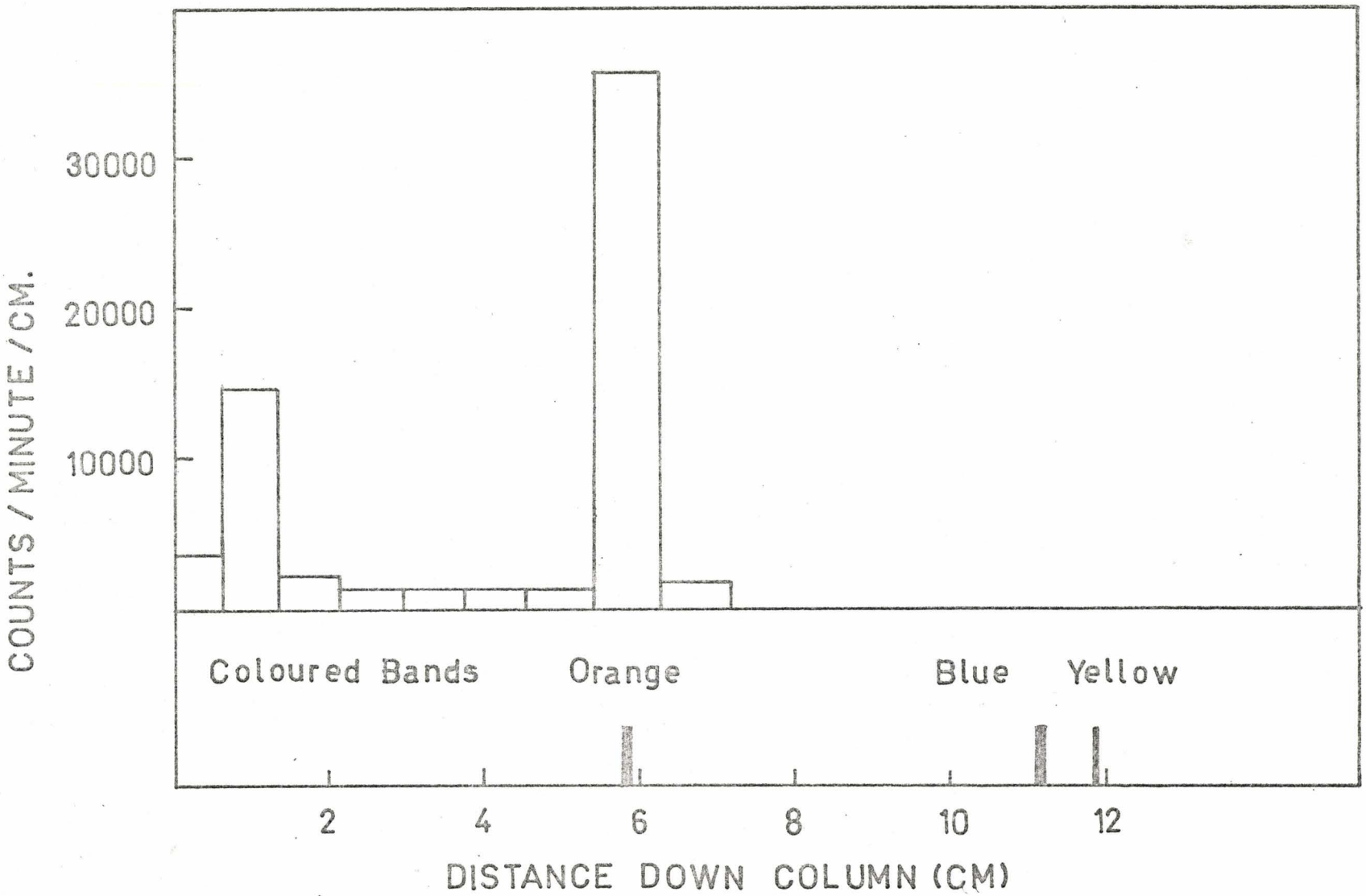

30000
20000
10000
COUNTS / MINUTE / CM.
Coloured Bands
Orange
Blue
Yellow
2
4
6
8
10
12
DISTANCE DOWN COLUMN (CM)

TABLE X

Disc gel electrophoresis of manganese-serum samples

Sample	Post-β Globulin Region		β Globulin Region		Cathodic Solution
	R_f max.	$\%^{54}Mn$	R_f max.	$\%^{54}Mn$	$\%^{54}Mn$
serum	0.07	34	0.54	46	20
	0.07	27	0.53	44	29
plasma	0.09	37	0.53	60	3
P-200 albumin fraction	0.08	32	0.54	60	8
	0.03	43	0.53	50	7
	0.04	32	0.53	55	13
	0.06	25	0.54	58	17
Averages		33±6		53±6	14±8

post- β globulin region can arise either from manganese-protein dissociation or from the existance of another manganese-protein complex. The latter situation is unlikely since, although the resolution of the radioactivity in the β globulin region was constant and equivalent to that of the protein present as shown repeatedly by protein staining, the shape of the electrophorogrammes in the post- β globulin region varied from run to run.

Samples of the manganese-54 - containing protein fractions, obtained in the P-200 gel chromatography of serum, gave essentially identical electrophorogrammes as those obtained with whole serum and plasma. A representative set of results is listed in Table X, page 62.

The average values for the manganese distribution in samples, without additives, were calculated using data from all electrophoretic runs performed, rather than from just those listed on Page 62.

In an attempt to establish the source of the manganese-54 in the post- β globulin region of the gel after electrophoresis, a slice of gel containing the β globulin fraction from a previous run was placed on top of a newly prepared gel column and electrophoreted. In the first trial, the gel block was placed on top after the new column had completely set, and air bubbles between the block and the gel were removed after addition of buffer. In other subsequent runs, the new gel was allowed to set

without the customary layer of water on top. When the gel was nearly set, the top quarter centimetre still being liquid, the gel block was carefully lowered unto this surface, air bubbles were removed, and the gel was allowed to set completely. Electrophoresis was then performed in the usual manner. The results of this work are listed in Table XI, page 65.

Once more, two manganese-54 bands were found in the gel, with the radioactivity distributed in the now familiar pattern. The low R_f values obtained for the β globulin band can be explained by a combination of two effects. The first is the effect of the previous history of the gel block being used. The two higher R_f values were obtained from gel blocks that were only a day old, whereas the lower R_f values came from blocks that were several days old, having to be rehydrated before use. Secondly, and more importantly, there was no albumin in the "gel block" electrophoreses. This meant that the standard blue albumin - bromo-phenol blue complex was not present, making it necessary that the purple free bromo-phenol blue band which marks the electrophoretic front be used as the calibration standard. This purple band, relative to the blue albumin-dye band, has an R_f value of 1.15±0.05. Accordingly, with this purple band as the standard, the β globulin species that formerly had an R_f value of 0.54±0.02 relative to the blue band, would have an R_f value

TABLE XI

Electrophoresis of β globulin-containing gel blocks

	Post-β Globulin Region	β Globulin Region		Cathodic Solution
	$\%^{54}Mn$	R_f	$\%^{54}Mn$	$\%^{54}Mn$
	33	0.43	47	20
	44	0.43	54	2
	44	0.49	51	5
	50	0.48	41	9
Averages	43		48	9

of 0.47±0.03.

The results show that the post- β globulin band arises from a concentration of ionic manganese-54 in this gel region. It is possible that either the nature of the gel region in contact with the cathodic buffer solution was altered in some manner relative to the bulk gel in such a way that it retarded the progress of cations through it, or that manganese ions had a low electrophoretic mobility in the gel.

To test the mobility of small quantities of ionic manganese through the gel column, samples containing manganese-54 were electrophoreted. When the manganese was applied to the cathodic end of the column, all but twenty per cent of the tracer remained in the cathodic solution. Of the remainder, more than ninety per cent was concentrated in the region within 1 cm. of the gel surface, even though the bromo-phenol blue indicator had travelled over 10 cm. in the same time. Addition of trace amounts of manganese to the anodic solution before exhaustive electrophoresis led to unexpected results. There was very little penetration of the gel by the manganese-54. Here, thirteen per cent actually penetrated the gel, but ninety per cent of this was in the first half centimetre.

Next, manganese-54 was added to the gel reagents so that gels containing a homogenous distribution of manganese were obtained. Bromo-phenol blue dye was added

to the upper reservoir, and electrophoresis was performed for varying lengths of time. The results are displayed in Figure III, page 68.

Exhaustive electrophoresis transferred the bromo-phenol blue marker dye completely from the cathodic to the anodic reservoir. In this case, radiomanganese was concentrated at both ends of the gel column and in the cathodic buffer. No radiomanganese was detected in the anodic solution. The congestion of manganese-54 in the cathodic region of the system arises from normal electrophoretic migration of manganese ions toward the cathode. But the presence of radioactivity at the anodic end of the gel can be explained either by the electrophoretic migration of anionic manganese (II) hydroxy species or by the formation of a complex between manganese ions and the marker dye. This complex would have to be stable enough to withstand electrophoresis, and yet dissociate in the region near the end of the gel column, since no radioactivity was found in the anodic solution. The second explanation was confirmed by electrophoreting for shorter times so that the bromo-phenol blue band remained in the gel after electrophoresis. (See Figure III, page 68) Manganese-54 was found to be associated with the indicator band. These results show that once manganese ions are released inside the gel column, their electrophoretic mobility is not as great as intuitively expected; therefore, concentration of

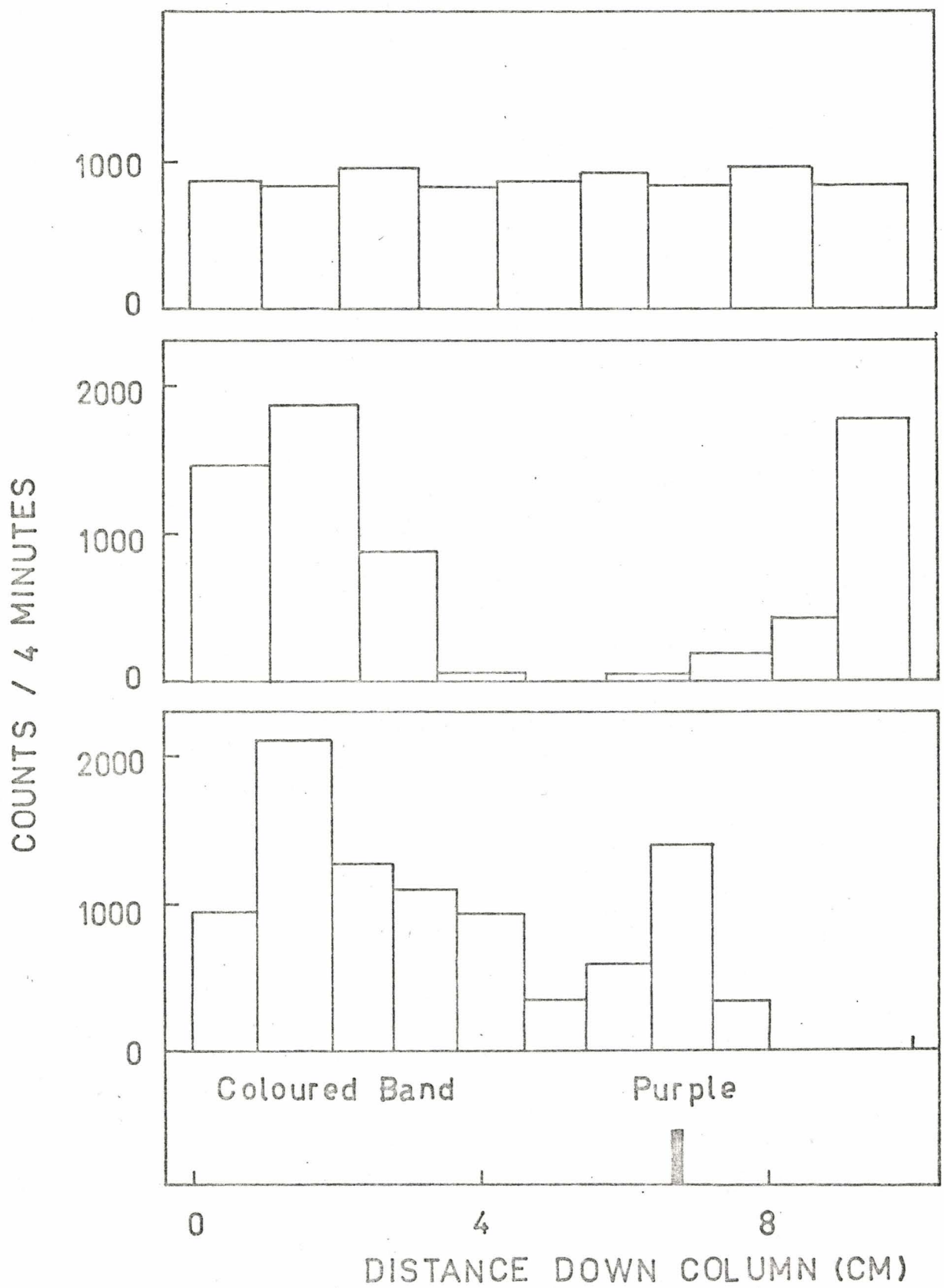
FIGURE III

Electrophoresis of Gels Containing Homogeneously Distributed Manganese-54

Upper: Homogeneous Distribution of Manganese-54 in Gel

Centre: Distribution of Manganese-54 Following Exhaustive Electrophoresis

Lower: Distribution of Manganese-54 Following Normal Electrophoresis


1000
0
2000
1000
0
2000
1000
0
COUNTS / 4 MINUTES
Coloured Band
Purple
0
4
8
DISTANCE DOWN COLUMN (CM)

the manganese-54 at the cathodic end of the gel would be predicted if a manganese complex were to dissociate within the gel. This process occurs in the case of the manganese-β globulin complex. The post- β globulin tailing observed upon electrophoresis of samples containing this complex is, therefore, certain to arise from this manganese ion migration phenomenon.

Addition of ethylenediaminetetraacetic acid to the system had the expected effect on the electrophoresis of radiomanganese. Electrophoresis after normal cathodic application of manganese, with $5x10^{-4}$M ethylenediamine-tetraacetic acid in the buffer, gave one band of manganese-54 activity with the electrophoretic front. After electrophoresis with anodic application, no radioactivity was found in the gel. Similarly, addition of ethylene-diaminetetraacetic acid to albumin fractions from P-200 gel chromatography just prior to electrophoresis drastically reduced the quantities of manganese-54 in the β and post- β globulin regions of the electrophorogrammes. Data are listed in Table XII, page 70. Though somewhat erratic, they show qualitatively the effects of the mixing time on the manganoglobin-ethylenediaminetetraacetic acid system. In these experiments there was an insignificant amount of radioactivity in the cathodic solutions, the ionic manganese being complexed by the ethylenediaminetetraacetic acid to form the $Mn(EDTA)^{-2}$ species which travelled with

TABLE XII

Electrophoresis of albumin fraction samples in presence of ethylenediaminetetraacetic acid

Mixing Time (min.)	Post-β Globulin Region		β Globulin Region		Electrophoretic Front
	Approximate R_f max.	$\%^{54}Mn$	R_f max.	$\%^{54}Mn$	$\%^{54}Mn$
EDTA layered above sample	0.09	10	0.54	81	9
5	0.10	1	0.53	62	27
10	0.08	13	0.53	51	36
10	0.10	26	0.54	14	60
10	0.08	27	0.52	47	26
15	0.08	3	0.54	3	94
15	0.07	2	0.53	2	96
15	0.08	13	0.54	6	81

the electrophoretic front. The presence of ethylenediaminetetraacetic acid did not influence the electrophoretic mobility of the radiomanganese-containing β globulin band.

Tables XI and XII illustrate that the electrophoretic mobility of the manganese-containing β globulin was constant, with R_f values varying by less than two per cent over many runs. The R_f values, averaged after twenty electrophoretic runs, was 0.53±0.01.

Rabbit transferrin (Pentex, RB3162, lot 5) was electrophoreted without further purification, and gave a major band in the β globulin region and two bands, which were hardly discernable after protein staining, in the post-β globulin region. The transferrin R_f value, relative to the purple bromo-phenol blue band, was 0.49±0.02.

This electrophoretic system fails, therefore, to separate transferrin, which is a β_1 globulin, from the manganese-containing β globulin.

The effects of magnesium (II) and calcium (II) on the disc gel electrophoretic behaviour of the manganese-binding β globulin were investigated in the light of the work of Foradori et al. Magnesium was employed to confirm its lack of effect on the system.[53] And calcium was used, since at least in paper electrophoresis it was found that addition of low concentrations of calcium (II) to the buffer solution resolved the globulins into β_1 and β_2(Ca) bands.[96] Table XIII, page 72, shows the results of this work.

TABLE XIII

Electrophoresis with Mg(II) or Ca(II) in the buffer

Metal	Metal Concentration in Buffer (mM)	Post-β Globulin Region		β Globulin Region		Cathodic Solution
		R_f max.	$\%^{54}Mn$	R_f max.	$\%^{54}Mn$	$\%^{54}Mn$
Magnesium	1.73×10^{-2}	0.04	43	0.53	50	7
Calcium	1.27	0.08	23	0.56	51	26
	2.20	0.08	26	0.53	61	13
	1.36	0.07	60	0.55	37	3

Laurell et al[96] found that calcium (II) in concentrations between 1.25 and 2.5mM had significant retarding effects on the β_2 globulins through the formation of a calcium-β_2 globulin complex. The β_1 globulins were unaffected in the system they used. In disc gel electrophoresis, with a calcium loaded buffer solution, there was a slight, but apparently insignificant, increase in the electrophoretic mobility of the manganese-54 - containing globulins. Therefore, based on Laurell's arguments, this β globulin is in fact a β_1 globulin.

The manganese-binding β globulin was electrophoretically unaffected by magnesium (II), even at concentrations far greater than physiological, a fact previously proven by Foradori et al, and confirmed here as an additional check of the manganese- β globulin system.

Serum was chromatographed on a P-200 column, and two adjacent fractions in the albumin region were then electrophoreted and stained to determine the protein distribution. One of these had 1.27mM calcium (II) in the buffer, and one had no calcium present. The R_f values of the stained protein bands are listed in Table XIV, page 74.

The predominant bands in these electrophorogrammes are those with R_f values of 1.00 (albumin - bromo-phenol blue complex) and 0.53 (β globulin). Unfortunately for the preceding argument, the presence of calcium in the buffer did not have a significant effect on the observed

TABLE XIV

Electrophoresis of albumin fraction samples in presence and absence of calcium (II)

No Calcium		1.27mM Calcium (II) in Buffer	
R_f max.	Relative Intensity	R_f max.	Relative Intensity
–	–	0.27	very weak
0.42	weak	0.41	weak
0.53	strong	0.54	strong
0.58	weak	–	–
0.68	weak	0.69	weak
0.75	weak	0.74	weak
0.79	medium	0.78	weak
0.83	medium	0.79	weak
1.00	very strong	1.00	very strong
–	–	1.10	weak
1.17	strong	1.19	weak

protein distribution. It could well be that the calcium (II) in the buffer was restricted from entry to the gel column in a manner similar to the restriction of the radiomanganese, thus preventing the calcium (II) from interacting efficiently with the protein sample during electrophoresis. To overcome this restriction, a modified gel was made that was approximately 2mM with respect to calcium chloride. Serum-manganese-54 solutions were elctrophoreted using this gel. The results are listed in Table XV, page 76, and again show the tendency for the elctrophoretic mobility of the manganese-binding β globulin to increase slightly, the average R_f value being 0.56. This small increase is not significant, since the range lies within experimental error.

The average electrophoretic distribution of manganese-54 in these calcium experiments is also recorded in Table XV. It is similar to that for electrophoresis in the absence of calcium as listed in Table X, page 62, and therefore confirms the lack of effect of calcium (II) on the relative manganese-54 distribution.

Four transferrin samples electrophoreted in gels containing 1.73mM calcium chloride produced orange bands with R_f values of 0.50, 0.49, 0.51, and 0.48 (average, 0.50). Once more, these were not significantly higher than in the "no calcium" case; therefore, the disc gel electrophoretic mobility of the β_1 globulin (transferrin) was indistinguishable from the manganese-binding β globulin.

TABLE XV

Electrophoresis using 5.5% gels containing calcium (II)

Sample	[Ca (II)] (mM)	Post-β Globulin Region		β Globulin Region		Cathodic Solution
		R_f max.	%^{54}Mn	R_f max.	%^{54}Mn	%^{54}Mn
serum+^{54}Mn	1.82	0.12	36	0.55	62	2
	1.82	0.12	32	0.57	66	2
	1.84	0.02	34	0.57	55	11
	1.88	0.05	31	0.57	58	11
	1.45	0.03	43	0.54	45	12
Averages*			36±12	0.56	54±10	10±8

* re Table XII and Table XIII as well

These electrophoretic experiments, in conjunction with Laurell's evidence on the retardation effect of calcium (II) on the electrophoretic mobility of $\beta_2$globulins, confirm that the β globulin which binds manganese-54 *in vitro* is indeed a β_1 globulin.

Consideration of both P-200 gel chromatographic and electrophoretic behaviour of this $\beta_1$globulin leads to the conclusion that it is remarkably similar to transferrin.

Cotzias was unable to detect any interaction between apotransferrin and manganese-54.[3] But, electrophoresis of a solution of rabbit transferrin - 2 mg./ml. in 0.15 ammonium acetate - with manganese-54 showed a considerable incorporation (thirty-four per cent) of radiomanganese in the $\beta_1$globulin region of the electrophorogramme. This shows that transferrin is quite capable of combining with manganese under *in vitro* experimental conditions. Therefore, there is no need to invoke the existence of a separate species to explain manganese binding by β_1 globulins in serum.

The effect of contamination of the manganese-serum system by foreign ions was studied by mixing small quantities of these ions with manganese-54 - labelled serum solutions, and then performing P-6 column chromatography. The results are listed in Table XVI, page 78. The metal ions were added in concentrations similar to the total metal ion concentration found in serum. This is probably

TABLE XVI

Contamination of manganese-serum system

Metal Ion	[Metal] Added ($x10^{8}M$)	Total Serum [Metal] ($x10^{8}M$)	Mixing Time (hr.)	$\%^{54}Mn$ Protein Bound
iron (III)	1.5	2.1	20	13
manganese (II)	0.14	$1.1x10^{-4}$	20	13
copper (II)	1.8	1.5	42	15
zinc (II)	1.5	1.5	42	10

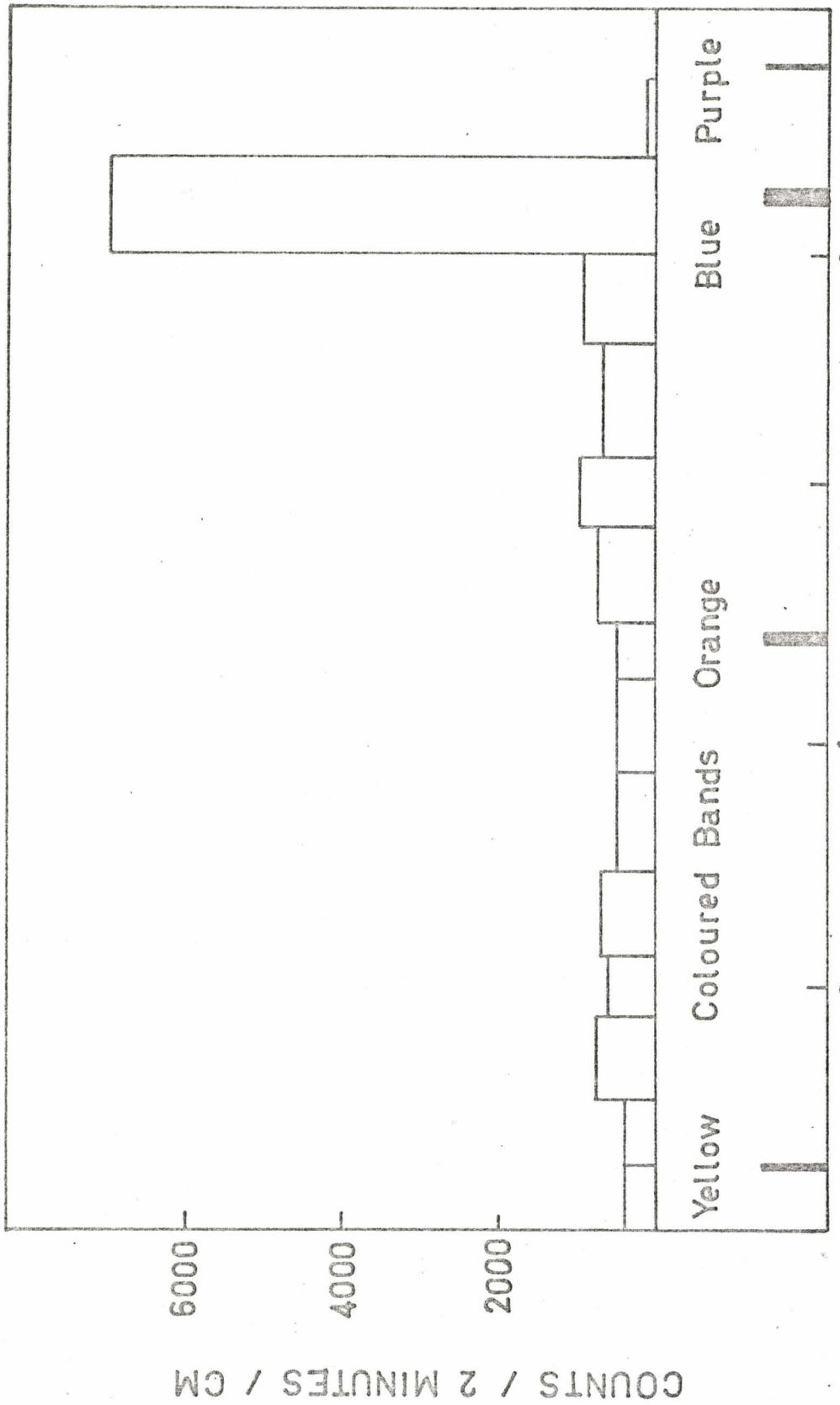
an extreme overloading of the system, but to make the required physiologically meaningful metal ion solutions with any hope of accuracy is impracticable.

The results show that contamination by microgram and sub-microgram amounts of metal ions can seriously alter the manganese-54 distribution. It is not even necessary that the metal ion bind specifically or strongly with the β_1 globulin that binds manganese-54, as is expected in the case of iron (III) and manganese (II). For example, very small quantities of copper (II) are bound *in vitro* specifically by the albumin, as shown by disc gel electrophoresis of human serum containing carrier-free copper-64 (see Figure IV, page 80), with no hint of any transferrin binding.

But use of these relatively high concentrations of copper reduced the manganese-54 binding capacity of the β_1 globulin in serum. If it serves no other purpose, this contamination experiment at least shows the need for very careful interpretation of experimental data. As an example, consideration of the similar effects of the copper (II), iron (III), and manganese (II), in conjunction with the reports of the *in vitro* synthesis of the copper (II)-transferrin complex[69], could easily lead to the erroneous conclusion that copper (II) binds selectively to transferrin, whereas the result recorded in Table XVI, page 78, arises from an overloading of the system with copper (II).

FIGURE IV

Disc Gel Electrophoresis of Human Serum Labelled *In Vitro* with Copper-64.
Sample size, 0.5 ml.


COUNTS / 2 MINUTES / CM
6000
4000
2000
Yellow
Coloured Bands
Orange
Blue
Purple
2
4
6
8
DISTANCE DOWN COLUMN (CM)

3.2. Manganese-Plasma System In Vivo

The short term incorporation of manganese-54 in blood has been studied extensively by means of injections of radiomanganese into rats and rabbits. Foradori *et al*[53] proved that manganese was bound selectively *in vivo* to a β_1 globulin in rat plasma. They allowed a radiotracer circulation time of four hours before exsanguination. Evans[70] injected manganese-54 into rabbits and used gel chromatography to separate the plasma proteins. He found that after a circulation time of twelve minutes manganese-54 was incorporated by a protein, or proteins, of molecular size similar to that of albumin. Following the work of Foradori *et al*, and Cotzias and Bertinchamps[95], Evans concluded that this was a β_1 globulin similar to transferrin. The studies of the *in vitro* manganese-serum system reported by Foradori *et al* and in section 3.1. confirm that this conclusion was justified.

However, Evans found that after several days a second manganese-binding protein could be distinguished, by P-200 chromatography, from the transferrin type protein which rapidly incorporated radiomanganese.

This second protein was shown to have a relatively high molecular weight which was estimated to be 200,000. The build up of radiomanganese in this protein was slow, and the metal protein complex formed appeared to be rather stable since it could still be observed after ninety days.

In order to gather more information about the high molecular weight manganese-binding protein, 0.8 mCi of carrier-free manganese-54 in sterile physiological saline solution was injected into a hooded rat ("Evans Strain") weighing 250 g. . A circulation time of six days was allowed to ensure that a significant uptake of manganese-54 by the high molecular weight protein had occurred. After this time the rat was exsanguinated by cardiac puncture, and the blood was citrated to prevent coagulation. The plasma solution obtained contained 500 counts per minute per ml. of manganese-54, as determined by the number 1. counting system described in section 2.2., page 37.

Plasma was chromatographed on a P-200 gel column (#47493), both in the presence and absence of ethylene-diaminetetraacetic acid. The results of these experiments are listed in Table XVII, page 83, together with those of the column calibration runs, and are displayed in Figure V on page 84. Only one protein region was associated with radiomanganese in each run. In both cases, this region corresponded to a protein of high molecular weight, since the elution volume of the radioactivity band was quite close to, though distinctly separate from, the void volume of the column. This meant that the protein molecular weight was higher than that of the largest protein (γ globulin) used in the calibration of the column. Hence, only an estimate of a minimum value for the molecular weight could

TABLE XVII

P-200 chromatography of *in vivo* labelled rat plasma and calibration proteins

Sample	Elution Volume / Void Volume	Band Name	Molecular Weight
Rat plasma	1.00	void volume	250,000
	1.63	γ globulin	160,000
	1.98	albumin	70,000
	1.16	^{54}Mn	200,000-250,000
Rat plasma + EDTA	1.00	void volume	250,000
	1.60	γ globulin	160,000
	1.94	albumin	70,000
	1.11	^{54}Mn	200,000-250,000
γ globulin	1.64		160,000
human albumin	1.96		70,000
egg albumin	2.26		43,000
cytochrome c	3.28		12,800

FIGURE V

P-200 (#47493) Gel Chromatography of
In Vivo Manganese-54 Labelled Rat Plasma.
Column Size, 33x1.6 cm. Sample size, 0.5 ml.

COUNTS / 50 MINUTES
2000
1500
1000
500
0
OPTICAL DENSITY
0.6
0.4
0.2
0.0
ELUTION VOLUME (ML)
10
20
30
40

be made. A value of 200,000 to 250,000 would appear reasonable, since proteins of molecular weight greater than 250,000 are eluted at the column void volume.

There was no indication of any radiomanganese in the albumin fractions after the chromatographic run. Therefore, the allowed *in vivo* cycling time must have been sufficient to remove the excess radiomanganese transported by the transferrin in the blood serum. The low specific activity of manganese-54 in the sample applied to the column, however, makes meaningless any estimate of an upper limit of the amount of radiomanganese in this chromatographic region, as well as in the ionic region.

The effect of ethylenediaminetetraacetic acid on the system was to reduce somewhat the level of radiomanganese associated with the high molecular weight protein, but it was unable to remove more than fifty per cent even after a mixing period of ninety minutes at room temperature (c.f. section 3.1.: in the *in vitro* system, mixing with ethylenediaminetetraacetic acid for sixty minutes removed 99.8 per cent of the manganese-54.)

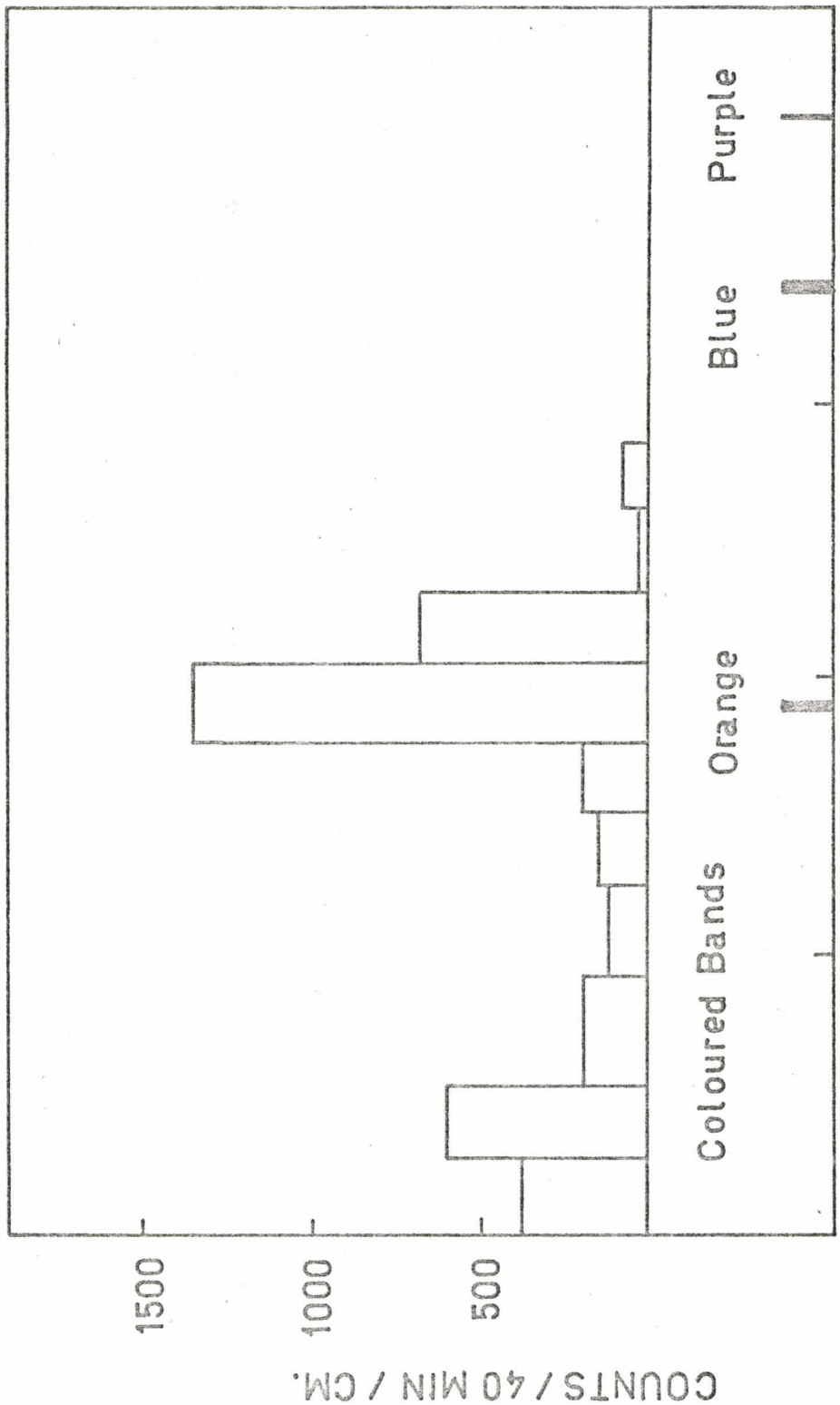
Six fractions containing the radiomanganese band, in the P-200 chromatography of plasma without ethylene-diaminetetraacetic acid, were combined into two fractions, and these were each disc gel electrophoreted. Because of the dilute nature of the protein solutions used in these electrophoreses, coloured bands were difficult to observe,

apart from the normal purple band of bromo-phenol blue indicator. No other bands were seen in the higher molecular weight fraction. A faint yellow band (R_f 0.54 relative to the blue dye-albumin band), however, was seen after electrophoresis of the lower molecular weight fraction. Since the amount of radiomanganese in the samples was rather low, long counting times were employed. The distribution of manganese-54 is displayed in Figure VI, page 87, the most salient feature being the lone radiomanganese band with an R_f value of 0.53 (average 0.51 and 0.55 in the two experiments). This R_f value is associated with β globulins (see section 3.1.). Therefore, the high molecular weight manganese-binding protein is a β globulin.

Three separate disc gel electrophoreses of whole plasma samples produced similar electrophorogrammes, containing manganese-54 bands with R_f values of 0.54, 0.57, and 0.54 (average 0.55). The last two electrophoretic runs were made four months after the first, and so were, in effect, a proof of the stability of the manganese-protein complex when stored at $0^{\circ}C$. After each electrophoretic run, three coloured bands were seen: firstly, a yellow band with an R_f value corresponding to that of the associated radiomanganese, and identified as the β globulin band; then the blue albumin-indicator band with its R_f value arbitrarily taken as 1.00; and finally, the purple bromo-phenol blue

FIGURE VI

Electrophoresis of the *In Vivo* Manganese-54 Labelled High Molecular Weight Protein Sample Following P-200 Gel Chromatography. Sample size, 0.5 ml.


COUNTS / 40 MIN / CM.
1500
1000
500
Coloured Bands
Orange
Blue
Purple
2
4
6
8
DISTANCE DOWN COLUMN (CM)

indicator band marking the elctrophoretic front.

Evans showed that it is extremely unlikely that the high molecular weight manganese-binding protein is a complex between haptoglobin and the manganese porphyrin-globin, similar to hemoglobin, from the erythrocytes. The low concentration of manganese-54 in the erythrocyte hemolysate, together with the pale pink-yellow colour of the plasma obtained from the rat, logically eliminates this possibility, unless the haptoglobin-manganese porphyrin-globin complex were exceptionally strong indeed.

The second possibility is that it is a trimer of transferrin. Although the polymerization of albumin has been established, no indications of polymeric transferrin have been reported. The observation of only a trimer, and not a mixture of trimer and dimer as in the case of albumin, is rather improbable. Also, the relatively small effect of ethylenediaminetetraacetic acid on the complex is not similar to its effect on the transferrin - manganese-54 complex which essentially can be totally dissociated. This fact may, however, not be relevant, since the spatial configuration of a polymeric protein may not necessarily be similar to that of its monomeric units.

Finally, the most probable explanation is that this manganese-binding protein is a high molecular weight monomer. The similarity of its electrophoretic behaviour to that of the monomeric, manganese-binding transferrin

supports this conclusion. This being the case, this high molecular weight manganese-binding protein shall be given the trivial name "plasmanganin", as recommended by Evans[70].

3.3. Distribution of Manganese in Erythrocytes In Vitro

Fresh human erythrocyte hemolysates were prepared for all experiments. The plasma was recovered from citrated blood after centrifugation, and the erythrocytes were washed repeatedly with 0.95 per cent sodium chloride solution. Hemolysate was then prepared as described in section 2.5. . The hemolysates were spiked with manganese-54. Protein fractionation and desalting were achieved by P-200 and P-6 chromatography, as well as by disc gel electrophoresis.

As in the case of serum, the amount of manganese-54 found to be protein bound was dependent on both the incubation time and the hemolysate preparation. In these experiments, the hemolysates were desalted using P-6 gel columns. The results are listed in Table XVIII, page 89.

When P-200 gel chromatography was performed, with blue dextran added to the sample solution to mark the void volume of the column, it was repeatedly found that more radiomanganese was associated with the blue dextran than with the red hemoglobin fractions, and that the sum of the manganese-54 in both regions of the chromatogramme constituted less than ten per cent of the total manganese-54 in the sample. The remaining manganese-54 was eluted at the total column volume as ionic manganese.

TABLE XVIII

Manganese accumulation by hemolysate proteins

Incubation Time	% ^{54}Mn Protein Bound
3 hours	0.3
3 days	14
6 days	18
11 days	36
14 days	39
5 minutes	12
2.3 hours	14
23 days	21

P-200 chromatography of hemolysate alone led to only one radiomanganese band in the protein-containing fractions. Thus, the radiomanganese found at the void volume was associated with the blue dextran itself, and not with cell debris or high molecular weight proteins. The lone manganese-54 band was not superimposed on the hemoglobin elution profile, but was eluted before the main red hemoglobin band (see Figure VII, page 91, and Table IXX, page 92). Results for the column calibration are listed in Table XVII, page 83.

The molecular weight of hemoglobin, as determined by this P-200 chromatography, is similar to the value of 64,450 calculated from amino acid analysis[135]. Therefore, the pH of the chromatographic system and the concentrations of hemoglobin used were such that very little dissociation of monomeric hemoglobin into the "halfmer" occurred.[43] Since no "halfmers" were detected, it seems very unlikely that dissociation occurred, and that this was followed by a recombination of a monomer with a "halfmer". This is the only hemoglobin-type species with a molecular weight around 100,000. The dimer, if observed, would have a molecular weight of nearly 130,000.

Another possibility is that a different protein species is responsible for the <u>in vitro</u> manganese-54 accumulation. Whatever the case, these experiments show that it is not the monomeric hemoglobin species that absorbs

FIGURE VII

P-200 (#47493) Gel Chromatography of *In Vitro* Manganese-54 Labelled Human Erythrocyte Hemolysate. Column size, 38x1.6 cm. Sample size, 1 ml.

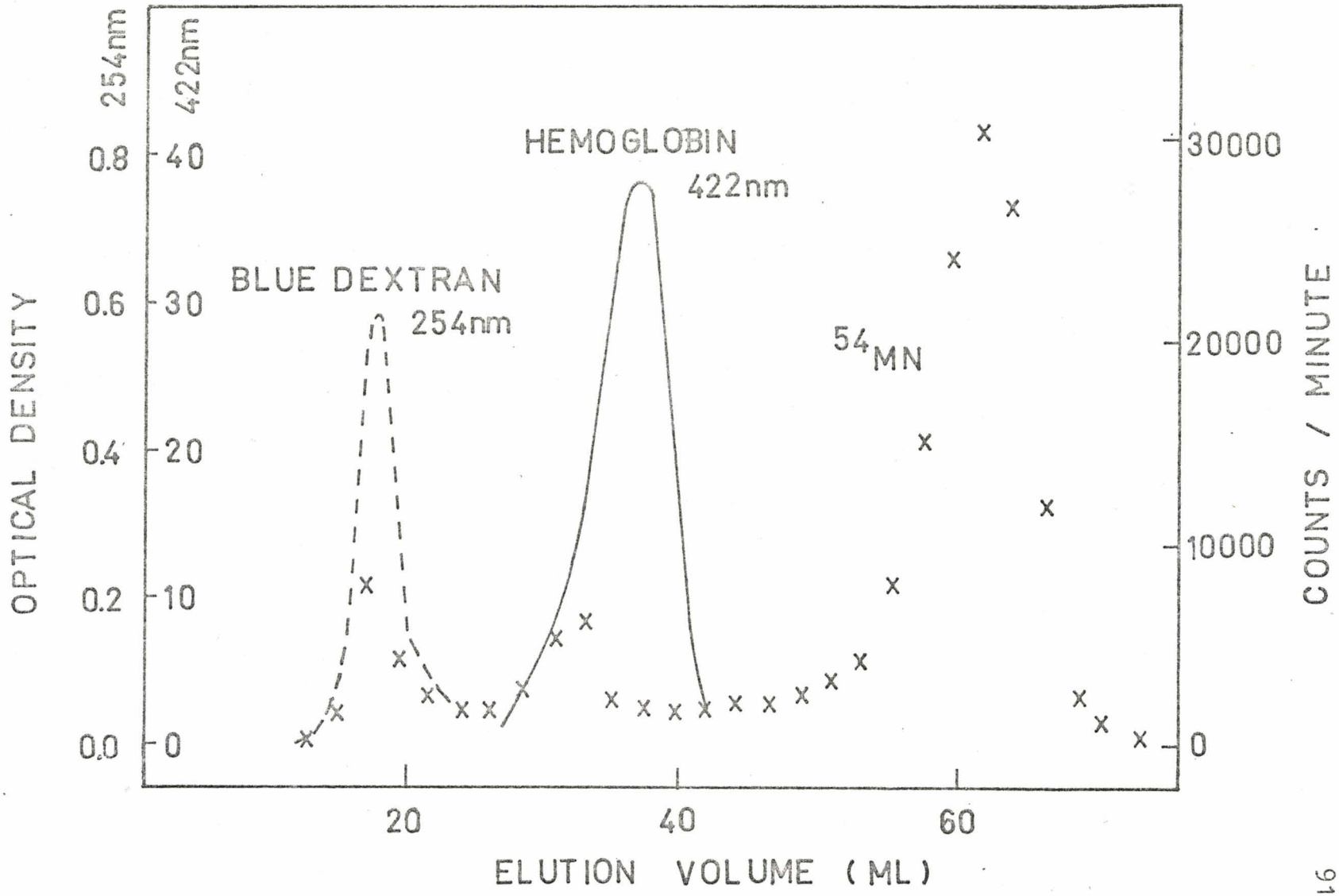

254nm
422nm
0.8
40
0.6
30
0.4
20
0.2
10
0.0
0
OPTICAL DENSITY
HEMOGLOBIN
422nm
BLUE DEXTRAN
254nm
54MN
30000
20000
10000
0
COUNTS / MINUTE
20
40
60
ELUTION VOLUME (ML)

TABLE IXX

P-200 gel chromatography of erythrocyte hemolysate with manganese-54

Species	$\frac{\text{elution volume}}{\text{void volume}}$	Molecular Weight
blue dextran	1.00	2,000,000
hemoglobin	2.02±0.03	63,000±3,000
manganese-54	1.00	250,000
	1.80±0.05	105,000±15,000

radiomanganese in the *in vitro* system.

Addition of ethylenediaminetetraacetic acid to spiked hemolysate led to a major reduction in the amount of radiomanganese associated with the hemoglobin molecules. In fact, an incubation of half an hour was sufficient to remove more than ninety per cent of the manganese-54 from the proteins after desalting on a P-6 column.

Because of the weakness of the manganese-54 - protein complex, electrophoresis of spiked hemolysate samples resulted in electrophorogrammes in which there was no large concentration of radiomanganese in any particular protein region. The radiomanganese was concentrated mainly in the cathodic solution and in the top third of the gel after electrophoresis. But some (less than ten per cent in each case) was found to be associated with the dark red hemoglobin band. Since ionic manganese does not penetrate very far into the gel upon electrophoresis (see section 3.1.), the radiomanganese found in the gel must have come from dissociation of the manganese-protein complex.

Premixing of the spiked hemolysate with ethylene-diaminetetraacetic acid of 10^{-3}M for a period of one hour removed essentially all the radiomanganese from the protein complex as evidenced by the fact that, upon electrophoresis, manganese-54 was found only at the electrophoretic front with the purple bromo-phenol blue indicator band. The added stress of charge transfer at the start of the electrophoresis may have aided the complete removal of

manganese-54, since when similar ethylenediaminetetraacetic acid-spiked samples were desalted on a P-6 column rather than being electrophoreted, approximately ten per cent of the radiomanganese remained in the protein-containing fractions, as previously mentioned.

Hence it has been shown that, although the in vitro addition of manganese-54 to hemolysate gave rise to some incorporation of radiomanganese by the proteins, the complex formed was rather labile. The work of Cotzias[106], and of Norris and Klein[111], is in agreement with this finding.

Initial studies of the competition of other metal ions for the manganese-54 binding positions were made by incubating small amounts of metal ion solution with desalted manganese-54 - spiked erythrocyte hemolysates which were then chromatographed on P-6 gel columns. The results for several metal ions are listed in Table XX on page 95.

A comparison of these results with those in Table XVI, page 78, shows the relative lack of effect the addition of metal ions has in the erythrocyte system. However, since manganese (II) has only slightly more success in replacing radiomanganese than the other metals, although it is there in relatively large excess, little can be gained by theorizing about the state of manganese-54 binding based on this work.

The most significant aim of the work with the in vitro system was to determine if the radiomanganese were

TABLE XX

Contamination of manganese-54 hemolysate with other cations

Metal Ion	[Metal] Added (M)	[Metal] in Erythrocytes (M)	Mixing Time (hr.)	$\%^{54}Mn$ Protein Bound
Fe(III)	1.5×10^{-8}	2×10^{-5}	20	70
Mn(II)	1.4×10^{-9}	1.1×10^{-11}	20	60
Cu(II)	3.6×10^{-8}	1.5×10^{-8}	24	69
Zn(II)	1.5×10^{-8}	1.5×10^{-7}	24	81

either bound to a protein molecule or incorporated in the hemin as suggested by Goldberg[112]. If the manganese was protein bound, then the acid conditions used to separate the heme from the globin would be harsh enough to completely dissociate the protein-manganese complex such that the radiomanganese should be found in the filtrate. Furthermore, if manganese-54 were associated with the heme, it should be recovered with the crystallized hemin.

To ensure that the system under study was the manganese-54 - protein one, spiked hemolysate was first desalted on a P-6 gel column to remove ionic manganese-54. Desalted hemolysate was then used in the preparation of hemin as described in section 2.5. The precipitated protein was washed three times with 0.1M hydrochloric acid in acetone, and this removed all but 0.2 per cent of the radiomanganese. Similarly, thorough washing of the prepared hemin removed all of the tracer which was subsequently recovered in the final filtrate. These results lead to the conclusion that the tracer was associated with the protein moiety and not with the heme.

3.4. In Vitro Manganese-54 - Hemin System

Because of the low solubility of hemin in aqueous solutions, it became necessary to investigate the suitability of non-aqueous solvents as elutants in a hemin chromatography system. This use of non-aqueous solvents in turn restricted the type of gel that could be used, the

common Bio-Gel P and Sephadex G series of gels being no longer practicable. A modified Sephadex G-25 gel - Sephadex LH-20 - which is operable in both polar and non-polar solvent systems, was tried, since the manufacturers claimed that separations of species on LH-20 columns was dependent essentially on the molecular sieving properties of the gel.

Since both polar (manganese (II)) and non-polar (hemin) species were to be separated without adsorption of one or the other on the gel beads, the choice of solvent appeared at first to be rather limited. It was decided to utilize the formation of pyridine hemochromes, if possible, since these are rather more soluble than hemin in both polar and non-polar solvents.

The following approach to the problem was taken: A pilot LH-20 column with a 12 ml. volume was set up and thoroughly washed with the solvent under investigation. A solution of hemin and manganese-54 in pyridine was then applied and eluted from the column. The elution positions and band widths of both species, and the column properties are reported below and in Table XXI on page 98.

Solvent 1 87 per cent chloroform : 8.7 per cent glacial acetic acid : 4.3 per cent pyridine.

The solvent mixture was denser than the gel beads, and this resulted in some mechanical problems in packing the column and maintaining it in a stable form. Acetic acid was added to discourage the formation of pyridine hemochromes. The

TABLE XXI

Chromatography of hemin and manganese-54 on Sephadex LH-20 with various solvents

Solvent	Column Volume (ml.)	Hemin		Manganese-54	
		Elution Volume (ml.)	Band Width (ml.)	Elution Volume (ml.)	Band Width (ml.
1.	12.0	7.2	2.0	10.3	3.0
2.	10.5	5.7	3.2	7.6	3.1
3.	13.0 (3 peaks)	5.3	6.0	16.0	adsorbed
		6.3			
		8.5			
4.	12.0	9.7	4.2	10.9	4.6
5.	31.7	18.8	5.0	22.3	5.0

bulk of the bright red hemin was eluted in a relatively narrow band, but there was a weak brownish coloured band which was eluted well behind this, together with some colouration of the fractions in front of the hemin. These secondary bands have been ascribed to contamination products. The manganese-54 elution maximum was clearly separated from the hemin band (see Table XXI, page 98), and 30 ml. of elutant was sufficient to remove all traces of manganese-54. Although the solvent performed as required, it was decided to try one of lower density to eliminate the problem of column instability. Accordingly, chloroform was omitted from the next solvent to be tried.

Solvent 2 80 per cent glacial acetic acid : 20 per cent pyridine.

After extensive washing, the column shrank to 10.5 ml. . In this system the hemin was eluted in a symmetrical band without any hint of tailing. But the colour of the band was not homogenous. The front edge was a yellow brown; the middle, a red brown; the rear, fading into pink. Also, the hemin band was much broader than that obtained in the chloroform-containing system, thus making the overlap with the added radiomanganese much more pronounced. Once more, the manganese-54 was totally eluted from the column.

An attempt to find a convenient elutant which reduced the spreading of the band led to the simple pyridine-water system described next.

Solvent 3 20 per cent pyridine in water.

The hemin was resolved into three distinct bands - a yellow band, followed by a red band, and then by a dark red one containing most of the hemin. Some manganese-54 was eluted after the hemin, but most was adsorbed near the top of the column.

A wash solution of 0.6M sodium chloride in the aqueous 20 per cent pyridine solution quantitatively removed the radioactivity, confirming the manufacturer's claim of strong cation adsorption on LH-20 gels at low ionic strengths, at least in the case of manganese.

Rather than use inorganic reagents in the system, acetylacetone was added, with the aim of complexing the ionic manganese and reducing its adsorption on the gel, and thereby giving rise to solvent 4.

Solvent 4 2 per cent acetylacetone : 20 per cent pyridine : 78 per cent water.

The hemin was eluted in a single wide band, the front and rear edges of which were yellowish, with a bright red maximum. Once more, the radioactivity was delayed on the column and was eluted in a single well defined band. There was, however, a trailing of a small amount of radioactivity, so it was decided that a less polar solvent should be tried.

Solvent 5 2 per cent acetylacetone : 20 per cent pyridine : 78 per cent methanol.

The hemin band had a much better resolution in this solvent

and the manganese-54 was eluted just as cleanly.

An attempt to calibrate the Sephadex LH-20 column with respect to molecular weight was then made. Fresh clover leaf extract gave rise to two bands upon chromatography - a green chlorophyll band, and a yellow carotene band. Hemin, manganese-54, riboflavin, and clover leaf extract were chromatographed separately and in suitable groups. In all cases, the elution volumes of each species remained characteristic. The average elution volumes are listed in Table XXII on page 102. Although this shows a trend towards proportionality between elution volumes of the species used and the molecular weight, major discrepancies occur. For example, the clover leaf chlorophyll and the bispyridine hemochrome are eluted at nearly the same volumes even though the molecular weights are distinctly different. Also, the riboflavin and bisacetylacetonato manganese (II) are eluted in the reverse of the expected order based solely on molecular weight considerations. Differential adsorption of the species on the gel matrix would tend to retard their elution from the column to different extents, and it follows that varying combinations of adsorption and molecular sieving are responsible for the LH-20 chromatographic behaviour observed.

At no time was any radioactivity found associated with the hemin band, even for mixing times up to a month.

TABLE XXII

Sephadex LH-20 chromatography in the pyridine-acetylacetone-methanol system

Species	Elution Volume (ml.)	Approximate Molecular Weight	Colour
clover leaf chlorophyll	18.4±0.4	910	green
bispyridine hemochrome	18.8±0.6	760	red
clover leaf carotene	20.7±0.5	540	yellow
riboflavin	24.3±0.6	380	yellow
$^{54}Mn(acac)_2$	22.3±0.7	250	colourless

A typical chromatographic elution pattern is displayed in Figure VIII on page 104.

3.5. In Vitro Manganese-54 - Hematin System

Dissolution of hemin in alkaline solutions produces a red-brown solution of hematin (hematoprotoporphyrin IX) in which the chloride of the hemin is replaced by a hydroxide ion. This is stored at 0°C in the dark to minimize the aggregation of the monomeric species to the β and ψ forms.[30] Non-aggregated hemin shall be termed hematin I, and aggregated hematin, hematin II. Hematin II is prepared from hematin I either by letting a solution of hematin I stand at room temperature in the light for a suitable time, or by heating it for short periods. Hematin II behaves gel chromatographically as if it were a species of specific molecular size (see below).

Various combinations of hematin I and II, and manganese-54 were gel chromatographed on a Bio-Gel P-6 column preequilibrated with 0.1M ammonium chloride buffer containing 0.01M ethylenediaminetetraacetic acid (see Table XXIII, page 105, and Figure IX, page 106). The ethylenediamintetraacetic acid was included in the elutant to remove from the hematin any loosely bound manganese-54. The elution volume of each species was characteristic and did not vary with the different mixtures. This indicates no association of the radiomanganese with either hematin species.

FIGURE VIII

Bispyridinehemochrome Spiked with Manganese-54 and Chromatographed on a Sephadex LH-20 Column (#1152, 62x0.8 cm.). Sample size, 1 ml.

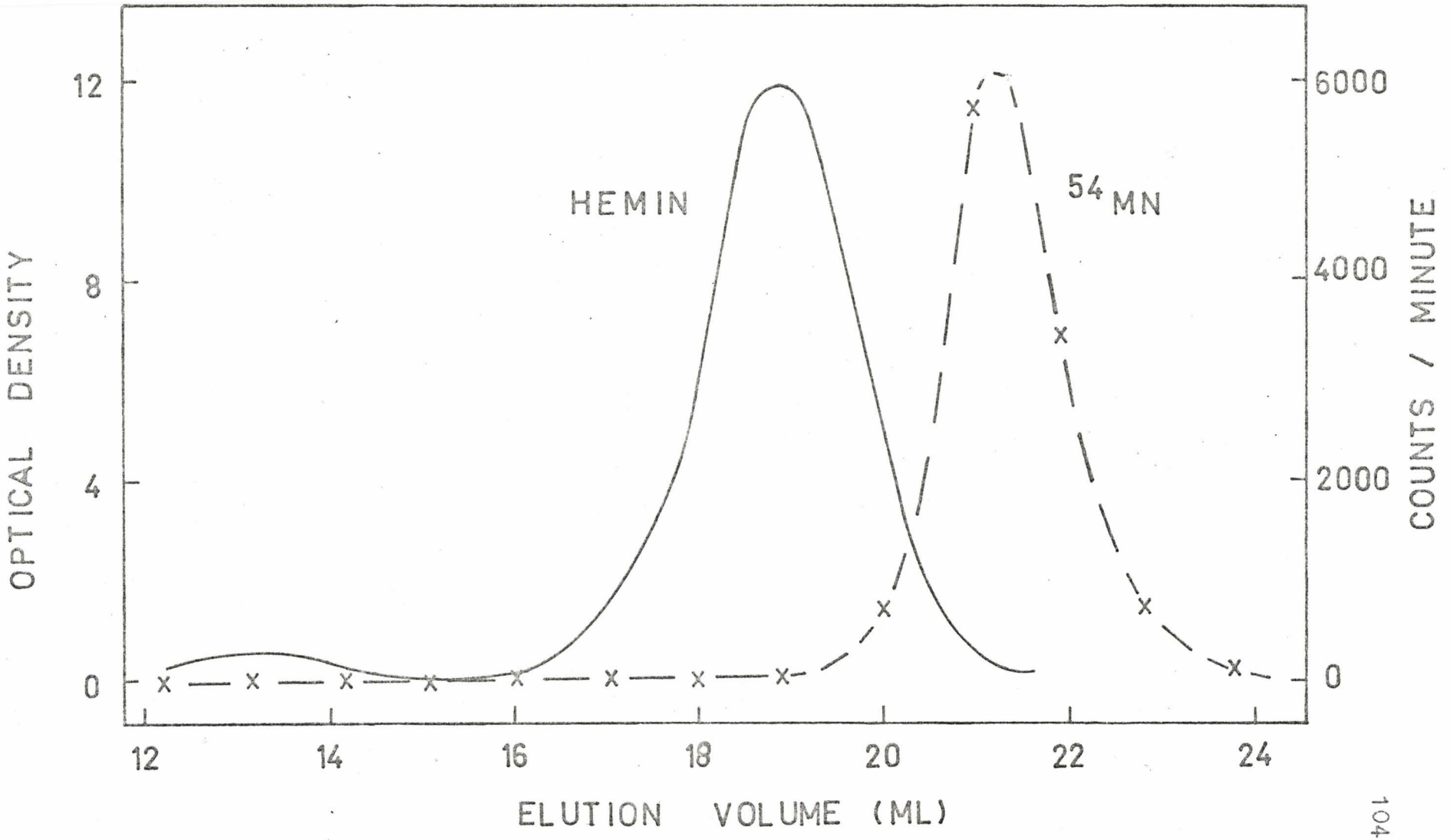

HEMIN
54 MN
OPTICAL DENSITY
COUNTS / MINUTE
ELUTION VOLUME (ML)
0
4
8
12
2000
4000
6000
12
14
16
18
20
22
24

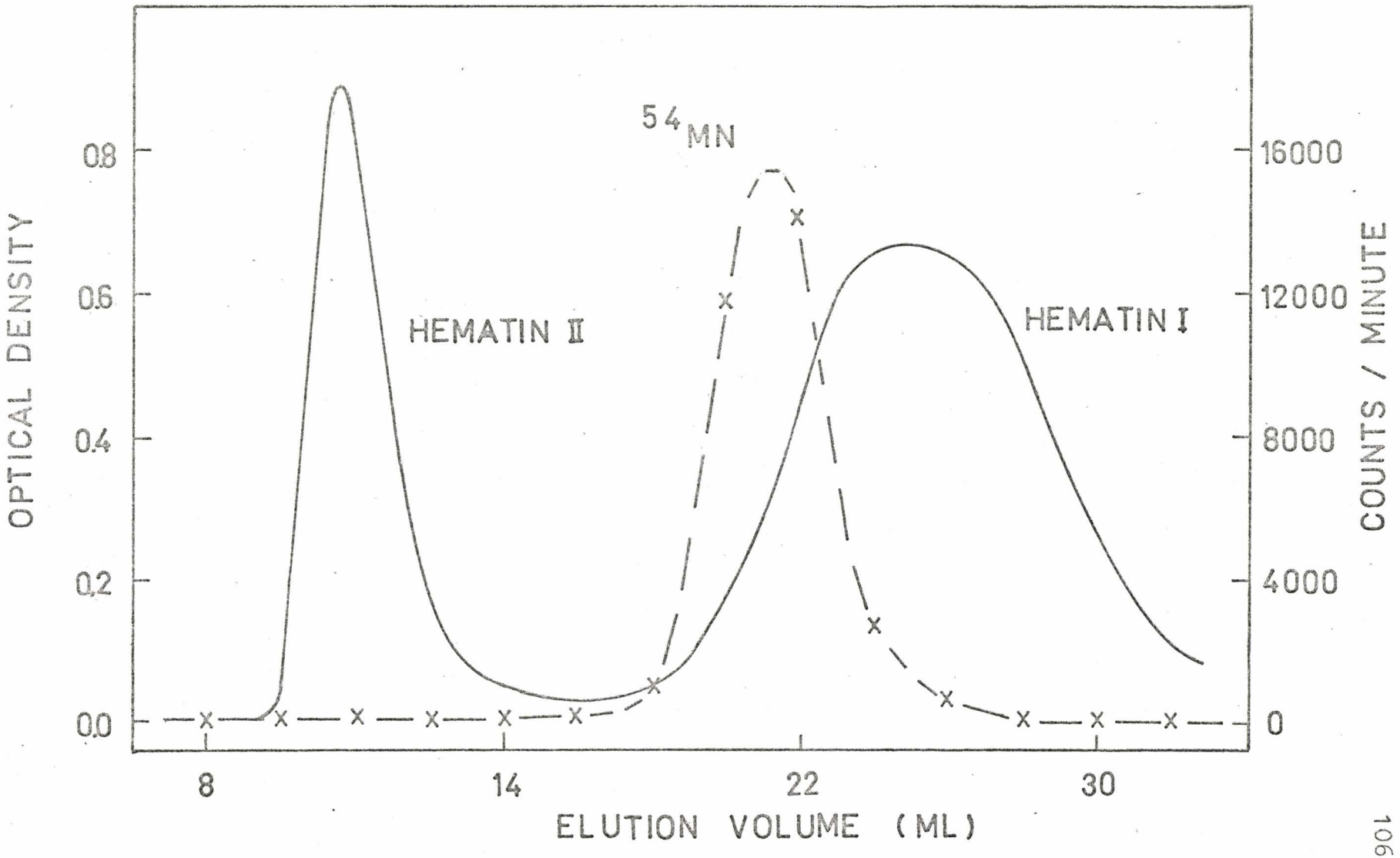
TABLE XXIII

Summary of gel chromatography with Bio-Gel P-6 at pH 9.4

Species	Elution Volume (ml.)
Hematin I	24.9±1.0
Hematin II	9.9±0.2
Manganese-54	21.2±o.1

FIGURE IX

P-6 (#54493) Gel Chromatography of Hematin I and II and Manganese-54 at pH 9.4. Column size, 16x1.8 cm. Sample size, 1 ml.


OPTICAL DENSITY
0.0
0.2
0.4
0.6
0.8
HEMATIN II
54MN
HEMATIN I
16000
12000
8000
4000
0
COUNTS / MINUTE
8
14
22
30
ELUTION VOLUME (ML)

In the P-6 chromatography experiments, hematin II was eluted at the void volume of the column. This indicated an aggregate weight of greater than 6,000. Evans, who worked with Bio-Gels up to P-10 in 0.1M ammonia, determined that the molecular weight should be greater than 10,000. Estimates, using other techniques, of between 30,000 and 90,000 have been reported[124,125], so use was made of Bio-Gel P-200 columns at pH 9.4. Once more, hematin I and II, and manganese-54 were chromatographed in different combinations, and no significant effects on the elution volumes were observed. (See Figure X, page 108) Various standard proteins were also chromatographed to calibrate the column. The average elution volumes are listed in Table XXIV, page 109. The aggregate weight of hematin was estimated, using the method of Andrews, by plotting the elution volume against molecular weight.[43] A value of 42,000±5,000 was obtained. This number is in agreement with the results of Gralen[124], and Shack and Clark[125].

The destruction of the Bio-Gel P-200 beads at pH 9.4 as a result of gel hydrolysis, followed by swelling and eventual blocking of the column, made it pointless to study the hematin II at higher pHs using this system. At pH 9.4 the average column life was between three and five weeks before the column properties changed significantly. Evans found that in 0.1M ammonia he barely had time to pack

FIGURE X

P-200 (#47493) Gel Chromatography of Hematin I and II and Manganese-54 at pH 9.4. Column size, 18x1.8 cm. Sample size, 1 ml.

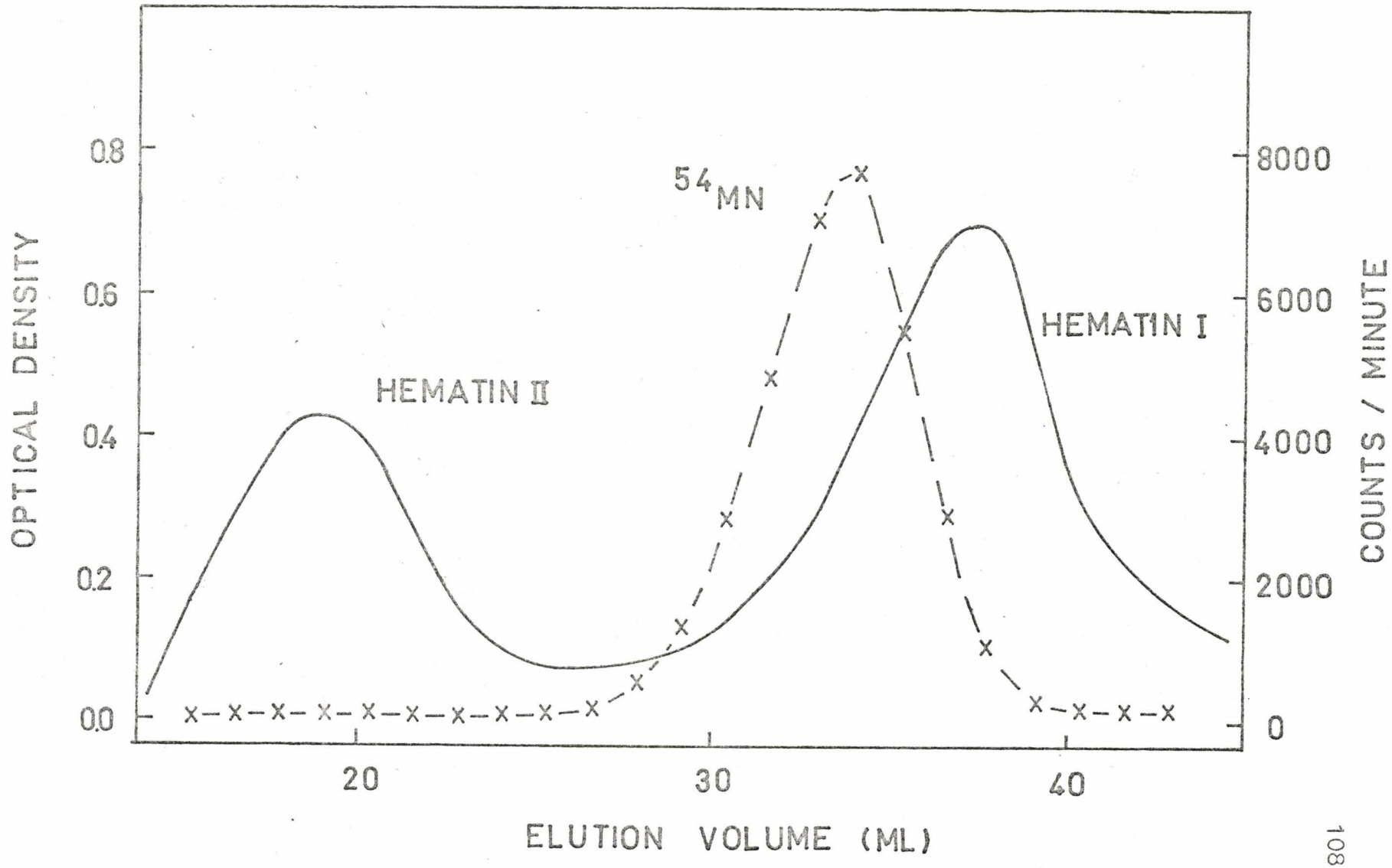

OPTICAL DENSITY
0.8
0.6
0.4
0.2
0.0
^{54}MN
HEMATIN II
HEMATIN I
8000
6000
4000
2000
0
COUNTS / MINUTE
20
30
40
ELUTION VOLUME (ML)

TABLE XXIV

Summary of gel chromatography with Bio-Gel P-200 at pH 9.4

Species	Elution Volume (ml.)	Molecular Weight
Manganese-54	34.3±2.0	
Hematin I	36.2±1.3	
Hematin II	18.9±1.2	42,000±5,000
Serum Lipoproteins	9.3±0.9	
Human Albumin	15.9±0.5	68,000
Egg Albumin	18.8±0.1	45,000
Cytochrome c	31.2±1.6	12,400
globulin	14.0	160,000

this type of column properly before gel swelling ensued.

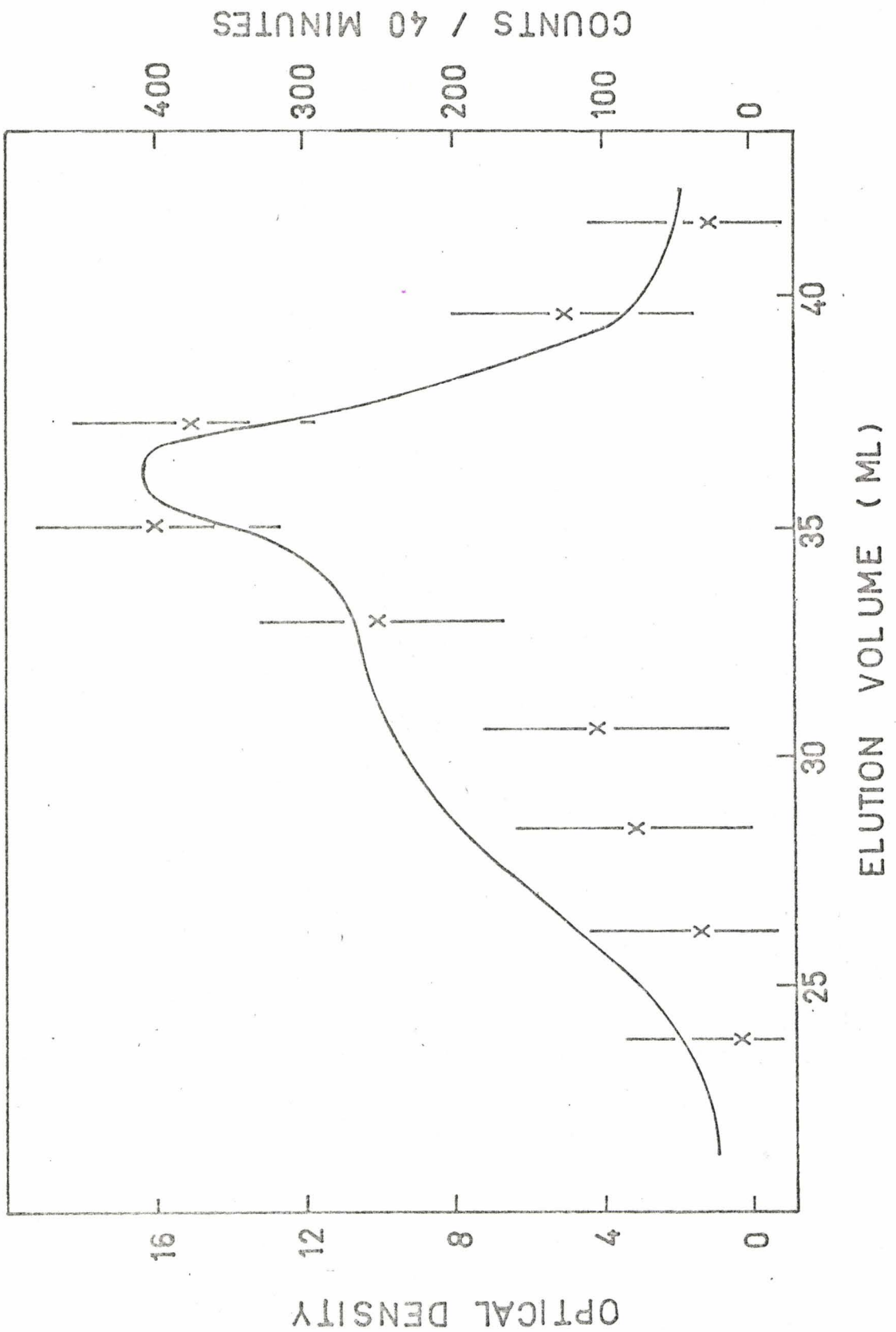
In both P-6 and P-200 chromatography, hematin I was observed to be eluted after the so called ionic manganese which at pH 9.4 is undoubtedly complexed with ethylenediaminetetraacetic acid, forming the anionic monoethylenediamine tetraacetomanganese (II) species. Since hematin I has a molecular weight of nearly twice that of the manganese-ethylenediaminetetraacetic acid complex, adsorption processes must be more dominant than molecular size effects in the elution of hematin I.

3.6. In Vivo Studies of the Distribution of Manganese-54 in Erythrocytes

Blood was withdrawn (see section 3.2.) from a hooded rat which had been injected six days previously with carrier-free manganese-54. Erythrocyte hemolysate was then prepared as described in section 3.3. The manganese-54 level in the hemolysate was low - 200 counts per minute per ml., using the number 1. gamma scintillation unit referred to in section 2.2., page 37. 1 ml. of this hemolysate was fractionated on a P-200 gel column (38x1.6 cm.), after incubation with 10^{-3}M ethylenediaminetetraacetic acid for thirty minutes, and the manganese concentrations in each fraction were determined by repeated counting. The results are shown in Figure XI, page 111. The distortion in the elution of the hemoglobin

FIGURE XI

In Vivo Manganese-54 Labelled Rat Erythrocyte Hemolysate Fractionated on a P-200 column (#47493, 33x1.6 cm.). Sample size, 1 ml.


COUNTS / 40 MINUTES
400
300
200
100
0
OPTICAL DENSITY
16
12
8
4
0
ELUTION VOLUME (ML)
25
30
35
40

band was a direct result of overloading the gel column by the use of too large a sample. This distortion did not affect the end result of the gel chromatography, since the radiomanganese was found to follow essentially the shape of the hemoglobin profile. Because of the low manganese count rates, and of the relatively large number of fractions which constituted the ionic band, no significant radiomanganese band was detected in this region of the chromatogramme.

The results of Borg and Cotzias[106] are in agreement with the above finding that manganese is indeed firmly bound in a hemoglobin-type protein in erythrocytes.

A 2 ml. sample of the in vivo manganese-54 labelled rat erythrocyte hemolysate was used in the preparation of hemin, by the method of Fritze and Robertson as described in section 2.5. . No manganese-54 was detected in the precipitated globin fraction after careful washing, even with prolonged counting. More than eighty per cent of the radioactivity was isolated with the crystallized hemin, the remainder being in the brown tinted supernatant. Further gentle evaporation of this solution led to the crystallization of more radioactive hemin, and to a consequent reduction of radioactivity in the supernatant to less than six per cent of the total. The colour intensity of the supernatant after the second crystallization was much less than before. This colour change, qualitatively correlated with the

removal of hemin from solution, together with the reduction in the amount of manganese-54 in the supernatant, confirmed the work of Borg and Cotzias who found that "the incorporated radiomanganese was isolated predominantly in the crystallized hemin"[106]. It also confirmed Evans' claim of a manganese-54 yield of more than eighty per cent in his hemin preparation.[70]

A portion of the rat hemin was dissolved in the 20 per cent pyridine - 2 per cent acetylacetone - methanol solvent, and chromatographed on a Sephadex LH-20 column (63.0x0.8 cm.). The chromatogramme is illustrated in Figure XII, page 114, and the results are listed in Table XXV, page 115. The bulk of the hemin (ninety-six per cent, from spectrophotometric measurements), which was bright red in colour, trailed a weak red-brown band. The separation of these two bands was quite clean. No radioactivity was detected in the front-running band which was most probably some hemin decomposition product. More than eighty per cent of the radiomanganese was concentrated with the hemin, the remainder appearing at the elution volume, associated with "ionic" manganese complexed by the acetylacetone.

In a second independent run, over ninety per cent of the hemin travelled with the bright red band, and more than seventy per cent of the radiomanganese was associated with this (see Table XXV, page 115). The remainder was

FIGURE XII

Rat Hemin Labelled *In Vivo* with Manganese-54 and Chromatographed on a Sephadex LH-20 Column (#1152, 62x1.8 cm.). Sample size, 1 ml.

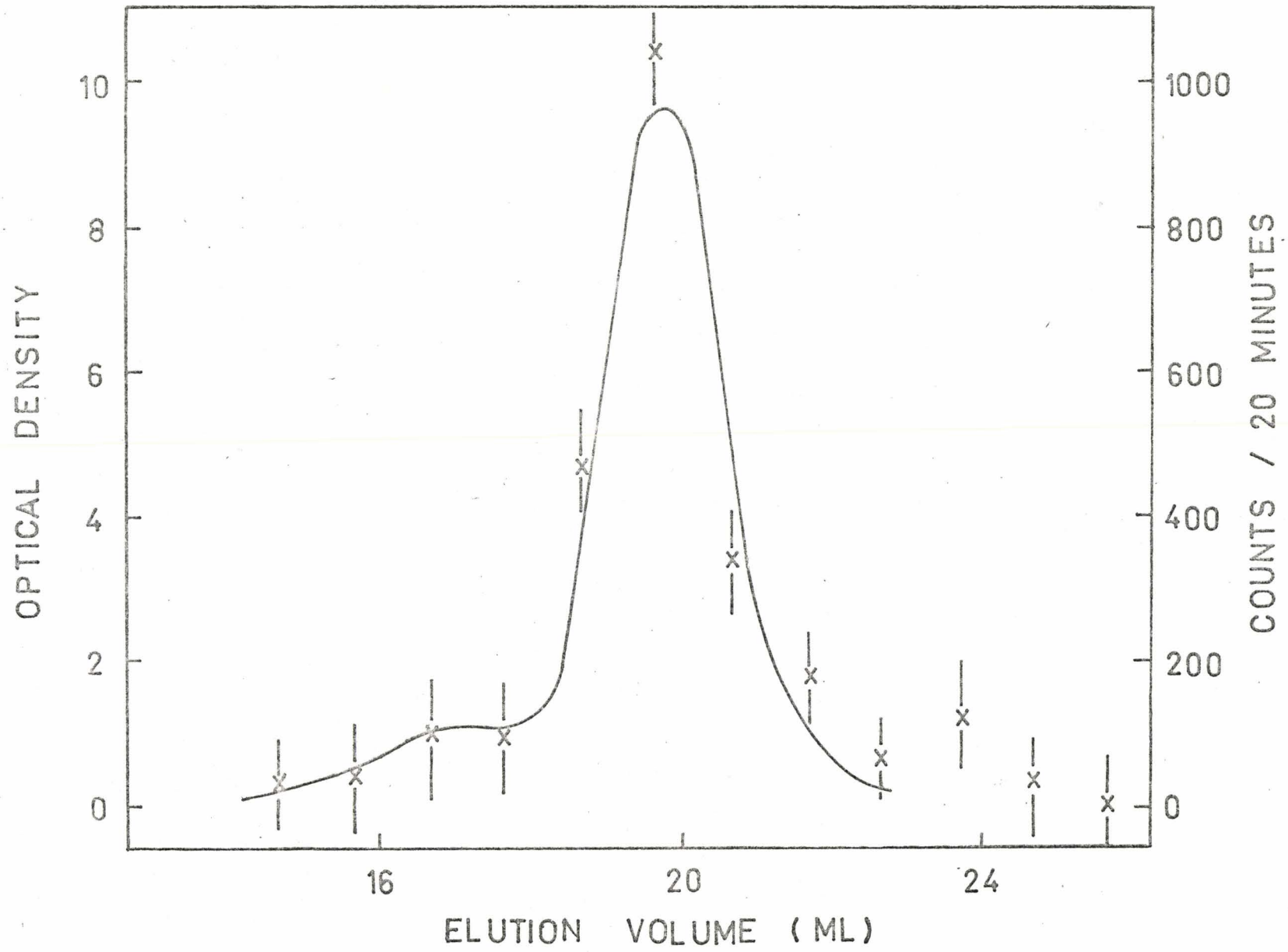

OPTICAL DENSITY
10
8
6
4
2
0
1000
800
600
400
200
0
COUNTS / 20 MINUTES
16
20
24
ELUTION VOLUME (ML)

TABLE XXV

Rat hemin chromatography using Sephadex LH-20

Sample	Coloured Bands			Associated Manganese-54 Band	
	Elution Volume (ml.)	Colour	%	Elution Volume (ml.)	%
hemin	15.9	red-brown	4		
	18.8	bright red	96	18.3	87±6
				22.7	13±6
hemin	15.8	red-brown	9	15.8	8±4
	18.8	bright red	91	18.8	73±
				22.7	9±5
evaporated hemin band	12.9	orange	4	13.0	11±1
	16.1	red-brown	8	16.3	9±1
	19.3	red	88	19.4	81±6
evaporated hemin band	15.7	red-brown	21		
	19.0	red	74	19.4	100
	22.4	yellow	5		
re-evaporated hemin band	14.7	orange-yellow	14	14.6	17±15
	19.0	orange-red	69	19.0	53±16
	21.7	yellow	17	21.7	30±17

eluted as "ionic" manganese. For each run, optical density measurements were made to determine the concentration of hemin and associated products in each fraction. Then, hemin-containing fractions were "pooled", and the total volume was reduced to about 1 ml., first by gentle heating, and then by vacuum evaporation. The results for the two samples prepared in this way, and then rechromatographed on an LH-20 column are also listed in Table XXV, page 115.

In the first case, there were three coloured bands: an orange band, a brown band, and finally a red one which contained eighty-eight per cent of the hemin products and eighty-one per cent of the radiomanganese. The other two coloured fractions contained the rest of the manganese-54. In the second chromatographic run, there were also three coloured bands. But this time, the order in which the bands appeared - the brown, the red, and then the yellow - varied because of differences in evaporation techniques. Nevertheless, although the red band accounted for only seventy-four per cent of the hemin, it still contained all of the radiomanganese. Accordingly, both runs indicated that most of the radiomanganese stayed with the hemin, even upon rather rough treatment. This conclusion was substantiated by the fact that hemin, reconcentrated from the two previous runs, when chromatographed for the third time still had fifty-three per cent of the radiomanganese with it in the red band.

A small displacement can be noticed between the experimentally determined elution volumes of some of the colour bands and the manganese-54 bands for any specific chromatographic run. These differences may be attributed to small errors in mensuration, and are therefore immaterial in any argument pertaining to the species in which manganese is bound in the porphyrin moiety.

Errors have been listed for the manganese distribution mainly to establish confidence levels in each set of results, and are based solely on counting statistics.

The change of colour of the various colour bands over this series of experiments is concomitant with a decrease in the quantities of manganese-54 present in each sample prior to chromatography, and is simply a matter of sample dilution.

A dark green-brown hematin II solution was prepared by dissolving crystalline rat hemin in a minimum volume of pH 9.4 buffer containing 0.01M ethylenediaminetetraacetic acid, heating the solution, and then adding extra buffer. This solution was then chromatographed on a Bio-Gel P-6 column (18x1.8 cm.). The results are displayed in Figure XIII, page 118, and Table XXVI, page 119. The hematin was eluted at the column void volume, and all of the radiomanganese was associated with the hematin-containing fractions. This is in agreement with the results obtained by Evans, who used 0.1M ammonium hydroxide as an eluant, and

FIGURE XIII

Rat Hematin II Solution Labelled *In Vivo* with Manganese-54 and Chromatographed on a P-6 Column (#54493, 18x1.8 cm.) at pH 9.4. Sample size, 1 ml.

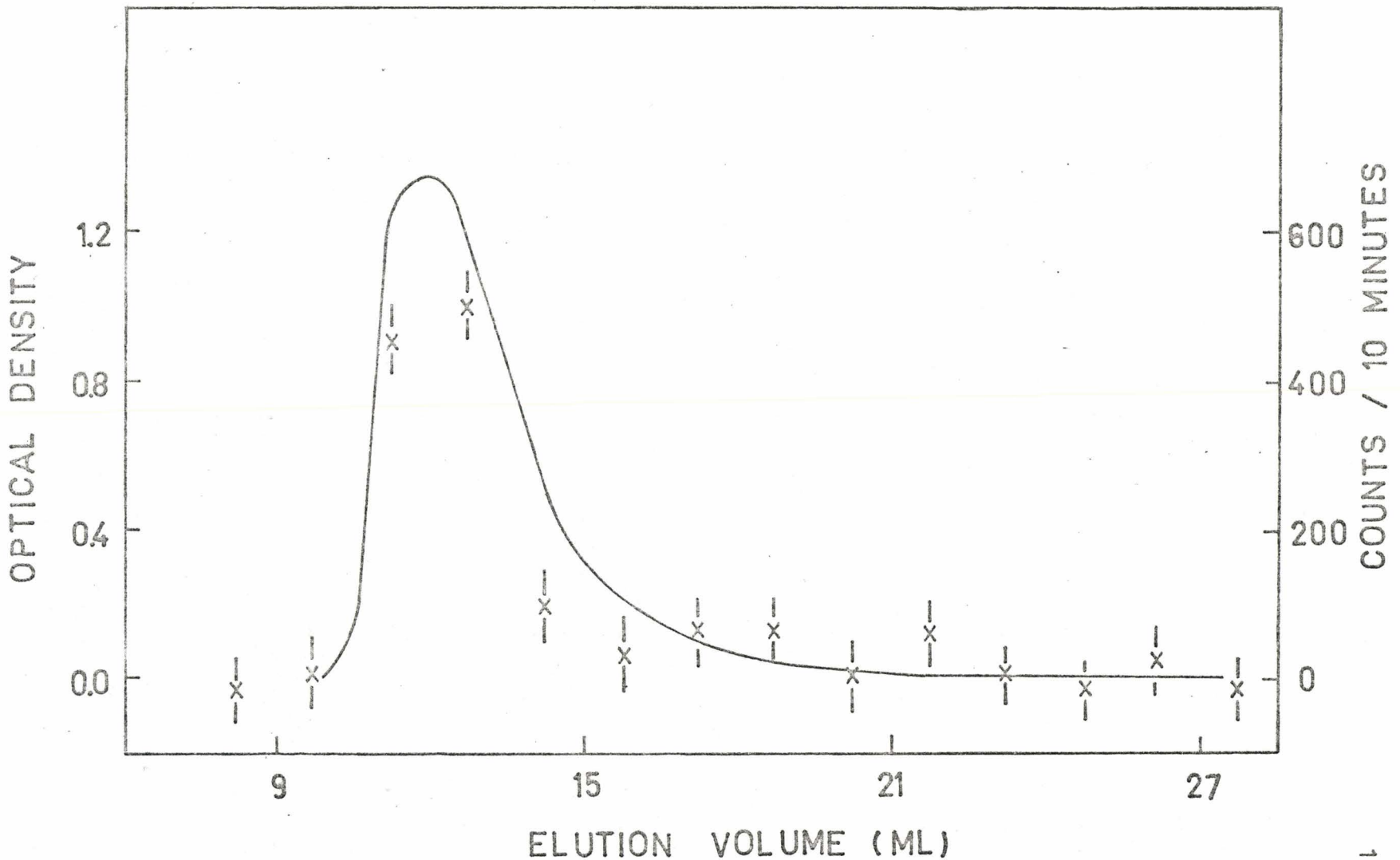

OPTICAL DENSITY
1.2
0.8
0.4
0.0
COUNTS / 10 MINUTES
600
400
200
0
9
15
21
27
ELUTION VOLUME (ML)

TABLE XXVI

Hematin II chromatography on Bio-Gel P-6 at pH 9.4

Sample	Species	Elution Volume (ml.)
rat hematin	hematin	11.8
	manganese-54	11.9
acetylacetone		28.2
pyridine		36.5
manganese-54		19.7
rat hematin from hemin	hematin	12.2
	manganese-54	12.1
	second U.V. active band	27.9
	third U.V. active band	36.5

confirms his report of polymerization of the manganese porphyrin, together with the hematin, in alkaline solution.

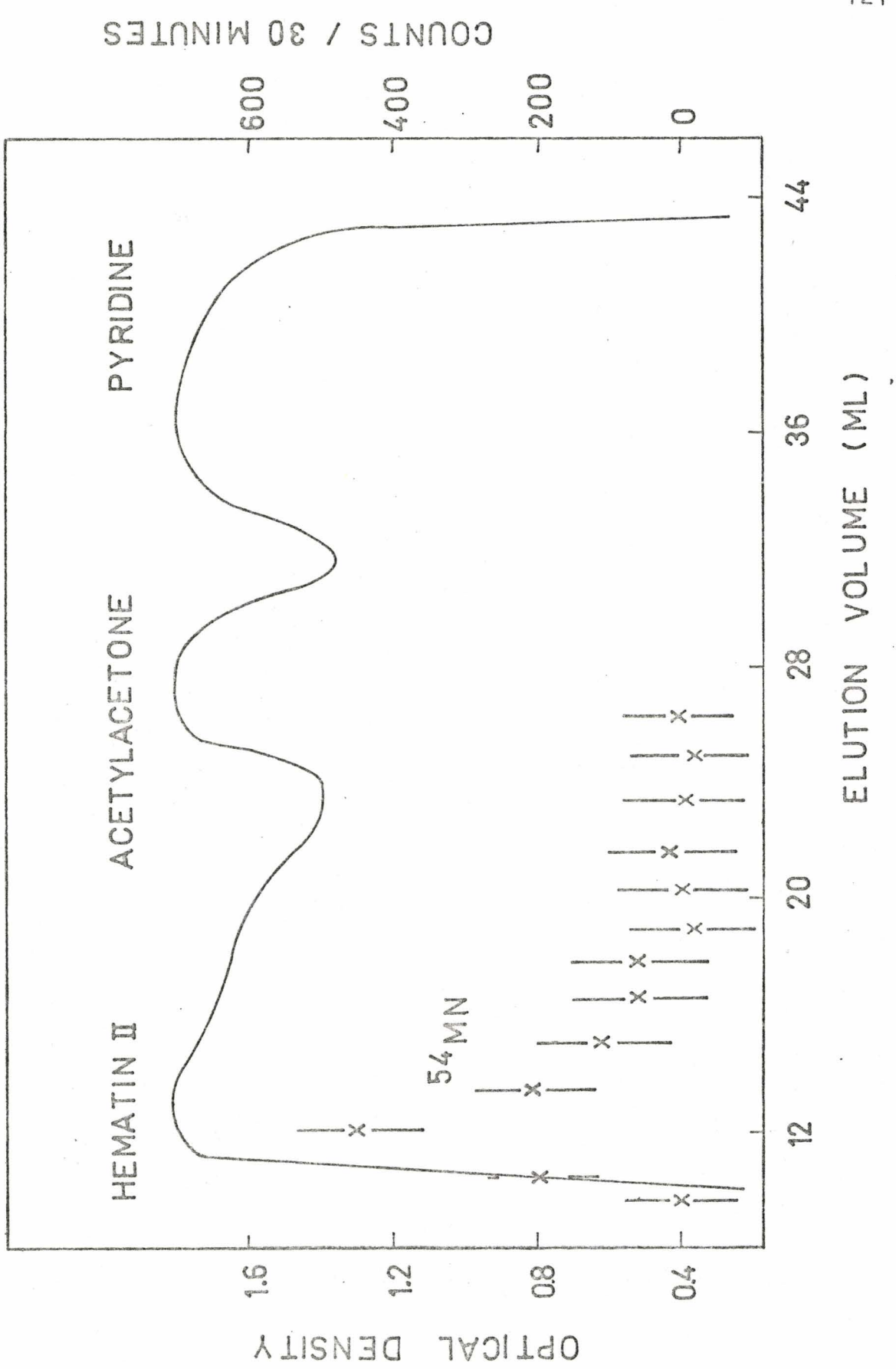
Attempts to convert the hematin fractions containing radiomanganese back to hemin by gentle evaporation and then dissolution of the resulting concentrate in the organic solvent gave rise to orange-brown rather than red-brown solutions. LH-20 chromatography of these samples led to inconclusive results, with the radiomanganese spread through the eluate and not specifically associated with either the hemin or the "ionic" manganese bands. Hemin decomposition products were present in major amounts.

However, the reverse procedure of converting hemin to hematin was much less physically aggressive towards the sample, and proved to be much more successful. Crystallized hemin was chromatographed on an LH-20 column, and the appropriate fractions containing the manganese-54 were gently heated to a minimal volume. pH 9.4 buffer was then added and the green-brown hematin solution obtained.

This was chromatographed on the P-6 column. The results are recorded in Table XXVI, page 119. and Figure XIV, page 121. Three bands which were optically active at 254 nm. were obtained. The first, which was green-brown in colour, contained the hematin; the second and third were both colourless. The third band corresponded to a set of fractions which had the characteristic odour of pyridine, and the second was odourless. Accordingly, the second band

FIGURE XIV

In Vivo Manganese-54 Labelled Rat Hematin II Chromatographed on a P-6 Column (#54493, 18x1.8 ml.) at pH 9.4. Sample size, 1 ml. (Hematin II was prepared from hemin following Sephadex LH-20 chromatography.)


COUNTS / 30 MINUTES
600
400
200
0
PYRIDINE
ACETYLACETONE
HEMATIN II
54MN
OPTICAL DENSITY
1.6
1.2
0.8
0.4
12
20
28
36
44
ELUTION VOLUME (ML)

was initially attributed to acetylacetone since this has a higher boiling point than pyridine, and both are present in the organic solvent used. Small quantities of acetylacetone and pyridine were chromatographed separately. Each was eluted as a single band which absorbed at 254 nm., and each corresponded to the appropriate anomolous band obtained in the hematin chromatography.

The radiomanganese followed essentially the same pattern as was found when hematin, prepared from crystalline hemin, was similarly chromatographed.

This series of experiments proves that the manganese-54 is incorporated in a porphyrin molecule, and is very firmly bound; that the manganese-containing species can be readily converted from the chloride to the hydroxy form with minimal breakdown; and that polymerization of hematin, containing the manganese porphyrin, results in the accumulation of the manganese porphyrin in the hematin polymer.

CONCLUSIONS

It has been shown here that a single addition of radioisotopes to blood, either *in vivo* or *in vitro*, may not necessarily disclose all the metal-binding proteins that are significant in the physiological state. To characterize all such proteins requires a combination of *in vitro* and *in vivo* studies. Furthermore, in *in vivo* experiments, circulation times of varying lengths are essential for differentiation between metal protein complexes that are formed rapidly and are responsible for the immediate transport of metal ions, and those complexes which can be detected only after some time in the living system and which play different physiological roles.

The effects of contamination on sample preparation have been shown repeatedly. Even with a minimum of handling and with stringent precautions against contamination being taken for each sample, the effects were at times disconcerting, especially in the case of erythrocyte hemolysates. Therefore, radioisotopic labelling of samples has a great advantage over other analytical techniques for determining relative manganese concentrations because of reduced sample manipulation before analysis. But even with this technique, quantitative estimates of the physiological manganese protein distributions cannot be made because of

the dissociation of manganese complexes induced by both random contamination and by the protein separation techniques employed.

In <u>in vitro</u> studies, only one manganese-binding protein was found. This has a molecular weight in the vicinity of 70,000, as shown by P-200 gel chromatography. disc gel electrophoresis experiments confirmed that it is a β_1 globulin. The manganese protein is relatively resistant to electrophoresis, and is stronger than the manganese citrate complex at the pHs used experimentally. But, it is less stable than the manganese-ethylenediaminetetraacetic acid complex. The radiomanganese in the complex can be exchanged by manganese-55.

This low molecular weight manganese-binding protein could not be differentiated from the protein transferrin using either gel chromatographic or disc gel electrophoretic techniques. It is therefore proposed that the name transferrin should be used to describe this manganese-binding protein, and that the name tansmanganin[95] is superfluous.

The relatively long term in vivo study of rat blood disclosed the other manganese-binding protein. This one had a molecular weight estimated to be between 200,000 and 250,000. It is analogous to the one found by Evans in rabbit plasma[70]. This high molecular weight manganese-binding protein is a β globulin, and is much

more resistant to ethylenediamintetraacetic acid than the transferrin-manganese complex. Since it was not observed in either *in vitro* or short term *in vivo* studies, this large manganese-binding protein should be considered to be a true manganese protein. It has been given the trivial name "plasmanganin".

The apparent incorporation of radiomanganese by the hemoglobin of erythrocytes *in vitro* is an absorption process in which a non-hemoglobin protein molecule and manganese ions interact relatively weakly.

On the other hand, *in vivo* incorporation of manganese-54 by erythrocytes resulted in the formation of one very stable species. The radiomanganese was isolated with the hemin in this case. Chromatography of hemin in pyridine-methanol solution gave no indication of separation between the bispyridinehemochrome and its radiomanganese equivalent. This indicates that the manganese is situated, in a manner similar to iron, in the centre of the porphyrin ring.

When rat hemin or the bispyridinehemochrome containing *in vivo* labelled manganese-54 was converted to hematin, and this was polymerized, the radiomanganese was found to be associated with this polymer. The polymeric hematin species, hematin II, has a discrete molecular weight determined by gel chromatography to be approximately 42,000 at pH 9.4.

The presence of a manganese porphyrin in the erythrocyte suggests that it is involved in the respiratory process, as is heme. Accidental replacement of iron by manganese during erythrocyte production and growth is most unlikely, since manganese is concentrated more in the erythrocytes than in the serum fraction, and it is via the serum that excess manganese is removed to the organs for storage or disposal.

In summary, in the physiological state there are two manganese-binding proteins in serum. The first is a β_1 globulin of molecular weight 70,000 which is remarkably similar to transferrin and which may act as a biological source of manganese. The second is a β globulin of molecular weight 200,000 to 250,000 that binds manganese very strongly.

In erythrocytes, however, manganese is predominantly bound to a porphyrin which is apoglobin-bound, giving rise to a species similar to hemoglobin.

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