COPPER, MANGANESE AND ZINC IN BRAIN

THE DETERMINATION OF COPPER, MANGANESE AND ZINC IN BRAIN TISSUE

By

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This project was undertaken with the aim of studying the distribution of copper, manganese and zinc in brain and to gain some information on the possible presence of soluble metallo-proteins in this tissue. The distribution of copper, manganese and zinc has been determined by neutron activation analysis. Soluble metallo-proteins of these three elements have been identified by the combination of gel chromatography and neutron activation analysis. In addition, in vivo and in vitro radioisotopic labelling techniques have been used occasionally.

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INTRODUCTION

1.1. General

Most of the elements in the periodic table have been found in the living organism. Among them, carbon, hydrogen, oxygen and nitrogen are known to be essential to all forms of life and contribute more than 95% of the mass of the organism. Sodium, potassium, calcium, magnesium, sulfur and phosphorus are less abundant and their concentrations are in the order of parts per thousand. Some elements, the concentrations of which are very low - even below one part per million - are called trace elements.

The role of trace elements in the living organism has also been studied. Many of them are believed to have some catalytic or structural function and be bound to macromolecules with precise molecular weights. These macromolecules are proteins and nucleic acids. It has been suggested that trace elements play a role in maintaining configuration of the protein and nucleic acid molecules and have a functional relationship to protein synthesis and the transmission of genetic information. However, for some of the trace elements, such as As, Sb, Cd, Au and Ag, there is no indication that they are involved in any vital processes.

The brain is the most complicated organ in mammals. It contains water (78%), lipids (10%), proteins (8%), inorganic salts (1%) and others (2%). It has significantly more lipid and less portein than most other organs. There are also differences between the chemical composition of grey and white matter. The concentration of some trace elements in the brain has been determined. In most cases, this involved the analysis of whole brain for the particular element. In the first in-

stance it is obviously of interest to determine whether the particular element is present at all. As soon as this is known, however, the question arises whether the trace element is evenly distributed. This is of significance in view of the anatomical and physiological complexity of the brain. A further decision can be obtained if one measures the subcellular distribution. Finally one would like to know in which form the trace element exists in different fractions. Apart from the purely analytical approach the injection of radioisotopes can in principle be used. However in this case one does not gain information on the "status quo" and is furthermore restricted by the blood-brain barrier.

The aim of the work presented in this thesis is to study the distribution of copper, manganese and zinc in brain and to gain some information on the possible presence of soluble metallo-proteins in this tissue. The distribution of copper, manganese and zinc has been determined by neutron activation analysis. Soluble metallo-proteins of these three elements have been identified by the combination of gel chromatography and neutron activation analysis. In addition radioisotopic labelling techniques have been used occasionally.

1.2. Trace Metals in Biological Tissue

The concentrations of trace metals in many different tissues have been determined by spectrophotometric techniques[1], atomic absorption spectroscopy[2, 3, 4], emission spectrography[5], neutron activation analysis[6, 7], and spark source mass-spectrometry[8]. Recently the distribution of 17 elements in subcellular fractions obtained from beef heart has also been measured[9]. The results were summarized by Bowen[10]

and by Underwood[11] who also evaluate published data critically. This is desirable as sometimes even very recent results are clearly too high by orders of magnitude[12]. Contamination is probably the most serious difficulty in the trace analysis of biological tissue.

Most of the trace metals are believed to be bound to proteins. Vallee[13] and Mahler[14] have differentiated two subclasses of proteins both of which contain metals. Their characteristics are listed in Table I.

A protein is a polymer of aminoacids. The groups available for binding to metals are -COOH, $-NH_2$, -OH, -SH from cysteine, =NH from histidine and $\geqslant$CH from tyrosine. Based on the chemistry of complexing agents, one may expect that Co, Cu, Fe^{II}, Mo, and Zn are most strongly bound by thiol, amino, or imino groups, with copper having the greatest affinity for both sulfur and nitrogen. On the other hand Ca, Cr, Fe^{III}, Mg and Mn should be most strongly bound by the carboxyl group or by any phosphate groups present. It is now clear that metallo-proteins containing copper, iron, molybdenum and zinc are numerous. Apart from the above, frequently, the metal is chelated by a small molecule called a "prosthetic group". Because of the extra stability of chelate formation many metals in metallo-proteins are bound to the phosthetic groups. One of the well-known metallo-proteins containing prosthetic groups is hemoglobin which accounts for 75% of the body iron. It contains four iron atoms (four prosthetic rings) with an atom of ferrous iron in the center of the porphyrin ring. Iron which has a coordination number of six is bound to four pyrrole nitrogens by ionic bonds and its two remaining valences bind to the protein by covalent bonds. Identification of the

TABLE I

Comparison Between Metallo-Proteins and Metal-Protein Complexes[13, 14]

Metallo-Proteins	Metal-Protein Complexes
1. Metal is firmly bound to protein.	1. Metal is loosely bound to protein.
2. Metal is unique.	2. Metal may be variable.
3. Metal to protein ratio is a small integer.	3. Metal to protein ratio is variable.
4. Metal to enzyme ratio is constant.	4. Metal to enzyme ratio is variable.
5. Enzyme activity disappears without metal.	5. Enzyme activity may exist without metal.

specific donor atoms of metal-binding sites in metallo-proteins lacking prosthetic groups is difficult. Generally, hydrogen ion titrations and chemical modifications of apoproteins have been used. The valency of the metal can also be determined by electron-paramagnetic resonance. Recently, an excellent monograph - "Metallo-proteins" written by Vallee and Wacker has been published[15].

Aside from proteins trace metals have also been found to be bound to nucleic acids. The binding sites in the nucleic may be phosphate groups, hydroxyl groups, and nitrogens from purine and pyrimidine bases. At the present stage, trace metals in nucleic acids have not been studied as extensively as in proteins. Most of the work has been carried out by in vitro experiments[16, 17, 18]. Although these experiments gave evidence for the complex formation between trace metals and nucleic acids, the conditions may be different from the actual physiological state in the living organism. On the other hand, iron has been found bound to DNA by an in vivo experiment in which radioactive iron was incorporated into DNA of rabbit liver[19]. By the combination of dialysis and emission spectrography, Wacker and Vallee have provided evidence for firm binding of chromium, manganese, nickel, iron and other metals in RNA from diverse biological sources[20]. Recently, the combination of dialysis and neutron activation analysis has provided evidence for tight bonds between gold, copper, manganese and zinc, and DNA molecules[21]. It was suggested that these metals play a role in maintaining configuration of the RNA molecules and have a functional relationship to protein synthesis and the transmission of genetic information. A review on the role of metals in nucleic acids and a theoretical study of metallic com-

plexes of nucleic acid have been published[22, 23].

Three methods can be used to study the metallo-macromolecules in biological systems. The first and second methods involve the use of radioactive isotopic tracers both *in vitro* and *in vivo*. From the analytical point of view, the use of radioactive tracer is the most convenient method to study the apparent distribution of metals in biological systems. However, the overloading effect must be considered in the *in vivo* experiments and the results from *in vitro* experiments may not reflect the true physiological state. The third method involves studying the distribution of naturally occurring isotopes *in vivo*. This method has been carried out by Himmelhoch *et al.*[24], (DEAE-cellulose chromatography-emission spectrography), Fritze and Robertson[25], Evans and Fritze[26] (Gel chromatography - neutron activation analysis). In the work presented here all three methods have been used in order to study the occurrence of soluble metallo-proteins of copper, manganese and zinc in brain tissue with particular emphasis on the analytical approach to gain information on the "status quo".

1.3. Copper in Brain

Copper is one of the most important trace elements in biological tissue. As early as in 1921, copper has been demonstrated in brain tissue[27]. So far, various analytical techniques have been used to determine the copper content in brain tissue[5, 28, 29, 30]. Practically all the work on brain deals with overall concentration, i.e. no attempts were made to distinguish between different regions of the organ. In some cases, however, the regional distribution was studied[31, 32].

Warren et al.[33] and Courville et al.[34] showed that the concentration of copper within the brain varies considerably with the anatomical location. Table II indicates that very large amounts of copper occur in substantia nigra and the locus caeruleus. Thompson[35] explained that presumably this unusually high concentration of copper in this area is related to its pigmentation. This pigmentation only occurs in old mammals.

The intracellular distribution of copper in brain tissue showed that 47% was in the supernatant fraction, 31% in the mitrochrondrial fraction, the rest in the combined nuclear and microsomal fractions; copper was absent in lipid extracts[36]. So far several copper proteins have been identified in brain tissue. They are cerebrocuprein, cytochrome oxidase, monoamine oxidase and dopamine β-hydroxylase. All of these are metallo-enzymes except cerebrocuprein. Cerebrocuprein has been isolated from brain supernatant[37, 38]. Recently this protein was proved to be identical with hepatocuprein and erythrocuprein; and all three have consequently renamed cytocuprein[39]. This copper protein contains 2 copper atoms per protein molecule with a molecular weight of 34,000. Cytochrome oxidase has been found in the mitochondrial fraction of brain tissue[40]. It contains one copper atom per protein molecule with a molecular weight of 93,000. Monoamine oxidase has been found in the mitochondrial fraction of brain tissue[41, 42]. It contains one copper atom per protein molecule with a molecular weight of 170,000. Dopamine β-hydroxylase has also been found in the mitochondrial fraction of brain tissue[43]. It contains two copper atoms per protein molecule with a molecular weight of 290,000.

Changes in copper content in brain tissue between healthy and

TABLE II[35]

Distribution of Copper in Normal Human Brain (μg/g dry wt.)

Region	Mean	No. of brains	Range
White matter			
Frontal lobe	12.8	7	10.0- 16.3
Occipital lobe	12.1	4	10.4- 13.2
Parietal lobe	11.3	6	7.4- 14.4
Temporal lobe	14.0	4	12.4- 16.3
Corpus callosum	9.2	5	5.9- 17.1
Internal capsule	13.2	5	10.5- 14.9
Base of pons	7.1	7	4.4- 9.9
Cerebral peduncle	12.7	5	9.1- 19.6
Cerebellum	13.5	7	5.6- 18.4
Optic chiasma	4.8	4	4.0- 5.4
Grey matter			
Frontal cortex	26.2	7	19.6- 32.6
Occipital cortex	23.9	6	13.2- 28.3
Parietal cortex	35.2	5	21.7- 50.3
Temporal cortex	26.6	5	23.0- 30.8
Caudate nucleus	29.4	6	19.8- 42.7
Putamen	32.9	6	23.3- 43.2
Globus pallidus	30.3	8	9.8- 42.8
Thalamus	16.0	7	13.3- 18.8
Subthalamic nucleus	21.5	7	9.4- 29.5
Mammillary body	23.7	9	12.7- 30.7
Red nucleus	22.7	3	21.3- 24.2
Cerebellar cortex	33.5	8	27.4- 39.0
Dentate nucleus	39.6	9	25.6- 59.1
Olive	22.0	4	11.0- 33.5
Substantia nigra	59.9	7	44.3- 76.9
Locus caeruleus	201.1	15	107.0-404.0

diseased individuals have been investigated. In the case of Wilson's disease - the failure to bind copper to caerulplasmin - copper is accumulated in liver and brain tissue especially in basal ganglia and cerebral cortex[44, 45]. The excessive accumulation of copper in brain tissue results in metabolic disturbance, because copper acts as an enzyme poison[46]. In studying cytocuprein in Wilson's disease and normal subjects, Porter found that the largest proportion of the brain tissue copper was in the soluble fraction with eighteen times more in diseased subjects. In DEAE-cellulose chromatography, soluble pathological copper was found reproducibly spread over a wide range of chromatographic fractions in diseased brain tissue, whereas, it formed reproducibly in a single peak in normal brain tissue[47].

The incorporation of radioisotopes of copper in brain tissue has not been carried out simply because of the short half-lives of ^{64}Cu and ^{67}Cu and the existence of the blood-brain barrier. This blood-brain barrier limits the penetration of radio-copper to brain so that most of the copper activity goes to other organs within the time limitations given by the half-lives of the available copper isotopes.

1.4. Manganese in Brain

Manganese, often resembling magnesium in enzymatic reactions, acts as an activator of many enzymes and this applies as much to the brain as to most other biological systems[48]. As an example, manganous ions have been found to stimulate the biosynthesis of cholesterol from acetate[49].

The element was found in normal brain tissue fifty years ago[50]. Methods including catalytic reactions[51], atomic absorption spectrometry[32],

emission spectrography[5], and neutron activation[6] have been used to determine the manganese content in brain tissue. Like copper in brain tissue, most of the analyses reported the overall concentration. However, Lechner et al.[52] found that the distribution of manganese is uneven throughout the brain.

Recently metallo-proteins of manganese in mammalian serum and erythrocytes have been identified[53, 54]. In contrast, no attempts have been made for the identification of manganese proteins in brain tissue. There are some hints that there may be at least one manganese protein in brain tissue. For one, the manganese content in brain tissue is relatively high in comparison to other organs. Also, manganous ions have been found to form metal-protein complexes upon addition of carrier-free manganese-54 to mouse brain homogenates[55].

Chronic manganese poisoning is a rare case of Parkinsonism - a brain disease[86, 87, 88, 89]. Degeneration of nerve cells is found throughout the brain but basal ganglia are most severely affected. Chronic manganese poisoning occurs among miners working with manganese ores[60]. The disease is characterized by a severe psychiatric disorder, followed by a permanently crippling neurological disorder clinically similar to Parkinson's disease.

1.5. Zinc in Brain

It has been known for a long time that zinc occurs in brain tissue[61, 62]. Topographic distribution of zinc has been found by atomic absorption spectrometry[63, 31], emission spectrochemical methods[34], and radioautography[64]. The results showed that the highest concentration is in the

Ammon's horn[65].

So far several zinc metallo-enzymes have been identified in the brain tissue. They are carbonic anhydrase, carboxypeptidase, glutamic dehydrogenase, lactic dehydrogenase, alcohol dehydrogenase, alkaline phosphatase, malic dehydrogenase and dipeptidase. Their molecular weights, zinc content and subcellular distributions are summarized in Table IV. Aside from these metallo-enzymes, another zinc protein has been identified in the soluble fraction of brain tissue[66]. It is cytocuprein which is usually thought of as a copper protein. Purified cytocuprein from human brain (among others) was found to contain 2 gram atoms each of zinc and copper per mole of protein. Upon dialysis against EDTA and 1, 10 phenanthroline, only relatively small amounts of zinc and copper could be removed from the protein.

Changes in zinc content in pathological brain tissue have not been studied sufficiently. In one case, a decrease in zinc content of brain tissue was found in postalcoholic human cirrhosis[67].

TABLE III

Zinc Metallo-Enzymes in Brain Tissue

Enzyme	Molecular weight	Amount of metal (%)	Ratio of metal to protein, g atom/mole	Subcellular distribution	Reference
Alcohol dehydrogenase	87,000	?	?	soluble fraction	68
Alkaline phosphate	?	0.15	?	nuclei	69, 70
Carbonic anhydrase	30,000	0.33	2	soluble fraction	71, 72, 73, 74
Carboxy-peptidase	34,000	0.18	1	mitochondria	75, 76
Dipeptidase	47,200	0.14	1	microsome	77, 78
Glutamic dehydrogenase	1,000,000	0.03-0.03	2-4	mitochondria	79, 80
Lactic dehydro-genase	137,000	0.07	?	soluble and microsomal fractions	81, 82
Malic dehydro-genase	40,000	0.14	1	soluble and mitochondrial fractions	82, 83

GENERAL EXPERIMENTAL PROCEDURES

2.1. Neutron Activation Analysis

Neutron activation analysis was first introduced by Hevesey and Levi[84] in 1936. The method was applied seldomly in early days. However, it has been one of the most useful methods for trace element analysis in the past ten years. This remarkable growth in application is attributed not only to the availability of high thermal-neutron flux reactors, but also to the development of radiochemical separation methods and radiation detection equipment.

The basic principle of the method is that stable isotopes, when irradiated by neutrons, can undergo a nuclear transformation to produce radioactive nuclides. The radioactive nuclides can then be determined qualitatively and quantitatively with or sometimes without chemical separations. Stable isotopes can undergo different nuclear transformations. The reaction which is the most important in trace analysis is neutron-gamma,

e.g. $^{75}As\ (n,\gamma)\ ^{76}As$

$^{23}Na\ (n,\gamma)\ ^{24}Na$

The induced activity (A, disintegration/sec) depends on the neutron flux (ϕ, neutrons/cm^2/sec), the thermal neutron cross section (σ, $10^{-24}/cm^2$), the number of target atoms present (N), the irradiation time (t) and the half-life of product nuclide ($T_{1/2}$).

The mathematical relationship between them is

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

Thus the sensitivity of the method depends on the above factors. For detailed discussion on the theory and practice one can refer to Lenihan and Thomson[85], Bowen and Gibbons[86], and Lyon[87].

Copper, manganese and zinc were studied throughout my work. Their nuclear properties are listed on Table 4. The analysis was based on the intensities of the 511 Kev line of ^{64}Cu, the 845 Kev line of ^{56}Mn, the 1115 Kev line of ^{65}Zn, and the 440 Kev line of ^{69m}Zn.

In a reactor there are several other nuclear transformations possible in addition to the (n, γ) reaction. The (n, p) reaction on iron and zinc will lead to high results of manganese and copper respectively if the sample contains large quantities of iron and zinc. Fortunately, there is a relatively high content of copper and manganese in brain tissue and the cross-sections of the (n, p) reactions of iron and zinc are only in the order of millibarns in the thermal neutron spectrum. Therefore, the contribution to the amount of copper and manganese on account of the (n, p) reactions of iron and zinc is negligible.

Neutron activation analysis is simple, sensitive and is possible to eliminate errors due to contamination by impurities in the reagents. By the addition of carriers after irradiation, the system is no longer microstage and conventional purification techniques can be used. Thus, the method has distinct advantages in the problem under consideration here: A sample size of 5 mg is sufficient for a reliable analysis. This means that small parts like the pineal body can be analysed. The

TABLE IV

Nuclear Properties of Copper, Manganese and Zinc

Stable isotopes	^{63}Cu	^{65}Cu	^{55}Mn	^{64}Zn	^{68}Zn
Percentage of abundance	69.1	30.9	100	48.9	18.6
Cross-section (barns)	4.5	2.3	13.3	0.47	0.1
Induced radioactive nuclides by (n,) reaction	^{64}Cu	^{66}Cu	^{56}Mn	^{65}Zn	^{69m}Zn
Half-lives of induced nuclides	12.8 hr.	5.1 min.	2.58 hr.	245 d	13.9 hr.
Type of decay	EC (42%) β^- (39%) β^+ (19%)	β^-	β^-	β^-	I. T.
Energy of gamma radiation (Mev.)	0.511 (38%) 1.34 (0.5%)	1.04 (9%)	0.845 (100%) 1.81 (22%) 2.11 (14%)	0.511 (3.4%) 1.115 (49%)	0.439 (95%)

concentrations of the three trace metals can be obtained from the same piece of tissue so that the ratios for different elements may be compared.

2.2. Gel Chromatography

The basic principle of gel chromatography is that different molecules, when passed through a porous granulated gel bed, can be separated according to their sizes. The pore sizes of the granulated gel regulate the size of the molecules that can penetrate through. As a result, a substance with a molecular size larger than the internal pores of the gel being used will pass through the column without diffusion into the gel and will be eluted at a volume equal to the void volume of the column. On the other hand, a substance with a molecular size smaller than the internal pores of the gel will diffuse into and out of those pores and will be eluted well after the void volume. Substances with intermediate molecular size can partially penetrate through the porous gel so that they will be eluted after the larger molecules but before the smaller ones. Thus, molecules can be separated in the gel-chromatographic column in the order of decreasing molecular size.

There are several important properties of the gel forming agent. Firstly, all ionizing groups must be absent. Secondly, there should be low affinity between the gel particles and the substances to be separated in a given solvent. Thirdly, the pore size of a gel must be controlled carefully. Finally, the gel particles should be as small as possible in order to establish diffusion equilibrium. In 1954 Deuel and Neukom[88] synthesized the first uncharged gel. Up to now, there are several

commercially available gels, namely, Sephadex (dextran gels), Bio-gel (polyacrylamide gels), Agarose (agarose gels) and Styragel (polystyrene gels).

The first theorectical approach to the specific behavious of gels in chromatography was studied by Flodin[89]. He considered the partition of the solute molecules between the gel particles and the interstitial liquid as an entirely steric effect. Based on this approach, the relation between the molecular weight of solutes and the partition coefficients has been studied[90, 91, 92]. However, this steric approach does not consider other factors especially the affinity of solutes to the gel phase. In general, the entropy part of the partition coefficient is contributed by steric effects, and the enthalpy part is due to the affinity of the solutes to the gel phase. For detailed theorectical discussion, one can refer to Determann[93].

One of the most distinct advantages of gel chromatography is the molecular weight determination. The results are in good agreement with other methods such as osmotic pressure and ultracentrifugation. The linear dependence of the elution volume on the logarithm of the molecular weight is used most frequently in practical works, i.e.

$Ve/Vo = k \log M$ Whitaker[94]

$Ve = k' \log M$ Andrews[95, 96]

Ve = elution volume of the particular solute

Vo = void volume of the column

M = molecular weight

k and k' = constants

Andrew has also shown that in the protein separation by gel chromatography this relationship holds only in the central region. At both high and low molecular weight ends the relationship does not hold.

In this work gel chromatography was used to identify the metalloproteins. The principle of the methods is as follows: The protein solution is fractionated on a gel column and the eluant is collected in small fractions. Each of those is analyzed quantitatively for the metal content. The column is also calibrated to obtain the elution positions of several proteins of known molecular weights. After all this data has been collected one plots metal concentration against molecular weight. If a "metal-peak" appears in the protein region a metallo-protein is assumed to exist. The peak position automatically gives a value for the apparent molecular weight. The main advantages of this technique are: one can "see" a metallo-protein in the presence of an almost unlimited number of other proteins since the identification is based on the metal rather than the protein part of the molecule. This type of chromatography is quite mild reducing the chance of liberating the metal from the protein. In fact, the pH is kept close to physiological conditions. Furthermore, one has a considerable choice for the eluting reagent. In this case ammonium acetate solution (pH = 6.9) was chosen because it can be obtained in very high purity and also its components do not in any way interfere in the subsequent metal analysis. In order to prove that the "metal-peak" represents a metallo-protein one performs an identical experiment after part of the original protein solution has been treated with an enzyme capable of hydrolyzing proteins. If now the

"metal-peak" is substantially reduced or preferably has disappeared altogether the justified assumption is made that the original peak was indeed due to a protein.

2.3. Materials and Reagents

Animal brains were obtained from Essex Packers, Hamilton. Human brains were obtained from Dr. A. Barbeau, Clinical Research Institute of Montreal.

In the dissection only plastic tools were used. They contained no detectable amount of copper, manganese and zinc.

All reagents were analytical grade except the chromatographic eluting solution - 0.15 M ammonium acetate (pH = 6.9). This solution was prepared by mixing Aristar acetic acid and Aristar ammonia. (B. D. H. Ltd., Toronto.)

The cellulose nitrate centrifuge tubes (2 x $\frac{1}{2}$ in) were supplied by Beckman Instruments Inc., California.

All the Bio-Gel P and ion-exchange resins (AG1 x 10, 100 - 200 mesh and Chelex 100, 100 - 200 mesh) were supplied by Bio-Rad Laboratories, Richmond, California. Bio-gel P, Bio-Rad's unique series of porous polyacrylamide gels, is made specifically for gel chromatography in aquous media. These gels are cross-linked copolymers of acrylamide and methylene bisacrylamide, produced in the form of spherical beads that can be hydrated readily in water or salt solutions. There are ten different Bio-gel P-X. X indicates that molecules with molecular weight greater than X,000 will be excluded from the gels. Three different gels

were used throughout my work, namely P-6 (#71803), P-10 (#37423) and P-100 (#3999 and #74643).

Blue dextran (Pharmacia, Sweden); human gamma globulins, bovine transferrin, bovin serum albumin (Pentex Biochemicals, III.); egg albumin, cytochrome C (Sigma Chemical Co. Miss.); and $^{24}Na^{+}$ were used for molecular weight calibration.

Two kinds of irradiation capsules were used. In the studies of distribution of trace metals in brain tissue, 1.7 x 1.4 (dia.) cm quartz capsules were used. They were immersed in aqua regia for 24 hours and then rinsed with deionized water. In the chromatographic experiments, polyethylene capsules with capacity of 5 ml were used. They were only rinsed with deionized water.

Two different kinds of standards were used throughout the activation analysis. In the determination of trace metals in brain tissue, a standard solution containing 0.10 μg copper, 0.0057 μg manganese and 0.19 μg zinc per milligram solution was used. About 20-30 mg (accurately weighed) standard solutions were dried in quartz capsules inside a clean desiccator. In the chromatographic experiments Alcan alloy 424 (3.7% Cu, 0.41% Mn, 0.98% Zn) obtained from Aluminium Company of Canada was used. Flux variations between samples and standards were monitored by 1% cobalt-aluminium wire.

Radioisotopes were mainly produced by the McMaster Reactor except ^{65}Zn (New England Nuclear Corp. Mass.) ^{24}Na was produced by irradiating a few milligrans of sodium carbonate using pneumatic rabbit system. ^{54}Mn was produced by the (n, p) reaction on spectral pure iron^{53}. The

following is an example for the production of carrier-free ^{64}Cu.

Carrier-free ^{64}Cu was produced by the (n, p) reaction of spectral pure zinc pellets. Normally a zinc pellet (~300 mg) was irradiated for twelve hours in a cadium envelope. After cooling for another twelve hours, the zinc pellet was rinsed briefly with 2 M HCl. Then, it was dissolved in ~3 ml concentrated HCl in a teflon beaker. The solution was evaporated to dryness under a UV lamp. The residue was dissolved in ~3 ml 0.01 M HCl. The solution was transferred quantitatively to a 4 x 0.6 cm Chelex 100 (100-200 mesh) column which was previously washed with 1 M HCl and 0.01 M HCl respectively. The column was washed with 0.01 M HCl until no detectable amount of ^{69}Zn was found in the washings. ^{64}Cu was then eluted with 5 ml 1 M HCl into a telfon beaker. The solution was evaporated to dryness under a UV lamp. After complete evaporation no detectable residue was found. At this stage, the gamma-spectrum was pure ^{64}Cu and the total activity was about 750 μCi. Different concentrations of hydrochloric acid were prepared by diluting Aristar HCl with deionized water.

2.4. Instrumentation

The 100,000 x g brain supernatants were obtained by using a Beckman Preparative Ultracentrifuge (Model L or L 2-65), rotors SW 50 or SW 65.

During gel-chromatographic fractionations, the eluant was monitored through a LKB Uvicord Absorptrometer(wavelength = 254 mu), and the eluant was collected fraction by fraction on a LKB ultra-Rac fraction collector. For the eluant subjected to subsequent activation analysis,

the chromatographic fractionations were carried out in a contaminant control box (Ultrasonic Industries Inc., N. Y.).

Irradiations for neutron activation analyses and production of radioisotopes were carried out in the McMaster Reactor with a thermal neutron flux of ~ 2 x 10^{13} neutrons/cm^2/sec and a cadium ratio of ~ 20. Irradiations less than 30 minutes were done using the pneumatic rabbit system.

The measurements of radioactivity were carried out by gamma-ray spectrometry, using the combination of the following detectors and single- or multi-channel analysers.

Detectors:

1. a 7.5 x 7.5 cm NaI(Tl) crystal
2. a 7.5 x 7.5 cm well-type NaI(Tl) crystal
3. a co-axial Ge(Li) detector with active volume of 12 cm^3
4. a co-axial Ge(Li) detector with active volume of 40 cm^3

Analysers:

1. a Nuclear Data Series 2200 1024 channel analyser
2. a Nuclear Data 256 channel analyser
3. a Victoreen linear amplifier (DD2, Model 851A) and a Nuclear Chicago scaler-timer

2.5. Procedure for the Avoidance of Contamination

1. Glass and quartz wares were immersed in aqua regia for 24 hours and washed thoroughly with deionized water.
2. Plastic tools and cellulose acetate ultracentrifuge tubes were

immersed in 2 M HCl for 24 hours and washed thoroughly with deionized water.

3. Sample preparations, extractions and chromatographic fractionations were carried out in a contaminant control box under positive air pressure.

4. Any metal contaminants in the gel columns were removed by washing with cyanide solutions. Prior to extractions and chromatographic fractionations, the metal contents in the extracting solution and the washing from the gel columns were examined by neutron activation analysis.

5. Internal contamination was eliminated by the addition of EDTA to the extracting solution.

2.6. Analytical Procedure for Copper, Manganese and Zinc in Brain Tissue

Brain samples were taken from the appropriate regions using only plastic tools. They were briefly rinsed with deionized water and transferred into quartz capsules. After freeze drying and weighing they were irradiated in the same containers. The samples, together with standards for Cu, Mn and Zn were irradiated for 6 hours. Separate experiments showed that the variation of flux between the sample and the standards never exceeded 5 per cent.

After a cooling period of 1 hour, the samples were mineralized with concentrated H_2SO_4 and fuming HNO_3 in the presence of about 2 mg each of Cu, Mn and Zn. The clear solution was diluted with water and neutralized

with NH_3 until the blue colour of the Cu-amine complex appeared. The final volume was about 10 ml.

At this stage the major activities are ^{24}Na, ^{42}K and ^{32}P. During preliminary work it was established that these interfering elements are most easily removed using Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.). Small columns (0.6 x 3 cm) of this resin (200-400 mesh) in the NH_4^+ form retain Cu^{2+}, Mn^{2+} and Zn^{2+} quantitatively at a pH about 8 whereas Na^+, K^+ and PO_4^{3-} run through and are completely removed by additional washing of the column with 5-10 ml of a 0.5 M ammonium acetate solution (pH = 8). The same separation can be carried out in the presence of a few drops of 5% (w/v) ammonium tartrate which prevents any precipitation of phosphates. The three elements are eluted together from the column with 1 M KCl into a 25 ml volumetric flask and made up to volume. The recovery is quantitative and consequently no chemical yield determinations are necessary. Both sample and standards were counted as solutions using a 12 cm^3 or a 40 cm^3 Ge(Li) detector in conjunction with a Nuclear Data 1024 channel analyser. The analysis was based on the intensities of the 511 Kev line of ^{64}Cu, the 845 Kev line of ^{56}Mn, and the 440 Kev line of ^{69m}Zn. Since the latter is usually quite weak the samples were recounted using a 7.5 x 7.5 cm NaI(Tl) detector after the complete decay of all short-lived activities. In this way the zinc was remeasured using the 1115 Kev line of the 245 day ^{65}Zn.

The three-element standard was leached out of the irradiation capsule using acidic carrier solution and the solution made up to volume

in a 25 ml volumetric flask. They were then counted under identical conditions as the sample. Half-life corrections were applied whenever appropriate. In preliminary calibration work it was shown that identical quantities of radioactivity gave the same count rates within 3% in the several 25 ml volumetric flasks which were used during the course of this work.

2.7. Extraction of Soluble Proteins

The soluble proteins were extracted from fresh beef brain tissue using five times its weight of a solution 0.15 M in ammonium acetate and 0.01 M in EDTA (pH = 6.9) at 4°C. After homogenization in an all glass tissue crusher the sample was centrifuged at 100,000 g for one hour. The supernatant and the pellet were freeze dried and analysed for copper, manganese and zinc by neutron activation analysis as described previously to obtain the percentage distribution between these two fractions. For the chromatographic experiments brain tissue was homogenized with only about half to one third its weight of the extraction solution. This ensures a sufficiently high metal concentration in the supernatant which is necessary for the analysis of the large number of fractions obtained after the protein separation. For *in vitro* experiments a drop of 0.15 M ammonium acetate solution containing either ^{64}Cu, or ^{54}Mn, or ^{65}Zn was added during homogenization.

2.8. Column Preparations

All gel chromatographic columns were prepared by using original dry

gel. For *in vivo* and *in vitro* experiments, the gel was swollen in the eluting buffer (0.15 M ammonium acetate solution, pH = 6.9) for 24-48 hours. The gel was added to a siliconized glass column and allowed to settle by gravity. The gel bed was then stabilized by washing with several column volumes of 0.15 M ammonium acetate solution of pH = 6.9.

If the eluant was subjected to subsequent metal analysis, the column preparation was different from those mentioned above. In this case quartz columns were used instead of glass columns. The quartz columns were first filled with aqua regia for 24 hours and rinsed with deionized water. The dry gel was swollen in 0.1 M ammonium-EDTA (pH = 6.9) for 24-48 hours. After settling by gravity in the quartz column, the gel was washed with about four column volumes of a solution 0.15 M in ammonium acetate and 0.01 M in sodium cyanide (pH = 7) to remove any trace metal contamination as completely as possible[26]. The column was then washed with about ten column volumes of high purity 0.15 M ammonium acetate of pH = 6.9. Columns prepared in such a manner were extremely "clean" because no detectable amounts of copper, manganese and zinc were found in the washing.

2.9. Protein Fractionation on Gel Column

In general, about 1.0 to 1.5 ml tissue extracts were fractionated. For easier application, the density of the tissue extract was increased by the addition of 10-20 mg sucrose. The tissue was then applied to the surface through a quartz pipette. After the tissue extract had reached 1 cm below the surface, the quartz reservoir was placed on top of the

column, and the elution was allowed to proceed at a flow rate of about 15-20 ml per hour. The eluant was also monitored through a LKB Uvicord Absorptimeter to give a rough protein elution curve.

If the eluant was subjected to subsequent activation analysis, the chromatographic fractionations were carried out in a dust-free box and the eluate fractions were collected in polyethylene capsules. The exact size of each fraction was determined by weighing, after which the contents were freeze dried. The polyethylene bottles were then heat sealed and ready for activation analysis. Immediately after chromatographic separations the columns were calibrated using blue dextrin, human gamma globulin, bovin transferrin, bovin serum albumin, egg albumin, cytochrome C and $^{24}Na^{+}$.

2.10. Enzyme Hydrolysis

To several grams of brain tissue extract an appropriate amount of either pronase or papain was added (a few mg). The almost clear solution was left at 40°C for a period between 24-72 hours. At the end of hydrolysis, the protein and ionic fractions were separated by the gel chromatography as described earlier.

2.11. Post Irradiation Treatment and Chemical Separations

All samples obtained from chromatographic experiments were irradiated in the dry state for two hours in the McMaster Reactor. After a cooling period of one hour the active material in the irradiated capsule was leached quantitatively with a hot concentrated nitric acid solution in

the presence of about 1 mg each of copper, manganese and zinc, and any organic material was mineralized. Evaporated to near dryness, the clear solution was diluted with water and neutralized with ammonia until the blue color of the copper-amine complex appeared. The final volume was about 2 ml. The solution was passed through a Chelex 100 column (0.6 x 3 cm, 100-200 mesh) and the column was washed with 5-10 ml 0.5 M ammonium acetate (pH = 8). At this stage, only Cu^{2+}, Mn^{2+} and Zn^{2+} were retained on the column. Since in these samples the activity of the three elements was considerably less than in the earlier work more detailed separations were necessary. In addition, new counting equipment became available while these analyses were in progress making further adjustments advantageous.

1. Eluted with 6 M HCl from the Chelex 100 column, Mn^{2+} was separated from Cu^{2+} and Zn^{2+} by passing through a Dowex AG 1 x 10 column (0.6 x 13 cm, 100-200 mesh) which was previously washed with 6 M HCl. Then, Cu^{2+} and Zn^{2+} were separated by eluting with 2 M HCl and water respectively. ^{56}Mn, ^{64}Cu and ^{69m}Zn were counted separately with a 7.5 x 7.5 cm NaI(Tl) detector in conjunction with a Nuclear Data 256 channel analyser. The counting efficiency could be improved by the precipitation of these ions on glass fiber filters as CuS, MnO_2 and $ZnNH_4PO_4$. The chemical yields were above 90%.
2. Eluted with 2 M HCl from the Chelex 100 column, Cu^{2+} and Mn^{2+} were separated from Zn^{2+} by passing through a Dowex AG 1 x 10 column (0.6 x 5 cm, 100-200 mesh) in 2 M HCl. Cu^{2+} and Mn^{2+} were collected in a 8 ml vial. Zn^{2+}, retained on the Dowex AG 1 x 10 resin, was

also transferred into a 8 ml vial in the presence of 2 M HCl. The recovery is quantitative and consequently no chemical yield determination are necessary. Both copper and manganese were usually counted together as solution with the 40 cm^3 Ge(Li) detector or occasionally with a 7.5 x 7.5 cm NaI(Tl) detector. Zinc was counted with a 7.5 x 7.5 cm well-type NaI(Tl) detector. The analysis was based on the intensities of the 511 Kev line of ^{64}Cu, the 845 Kev line of ^{56}Mn and the 440 Kev line of ^{69m}Zn.

The standard (Alcan alloy 424), dissolved in a few millilitres of aqua regia, was diluted to appropriate volume. The standards of copper, manganese and zinc were prepared according to the above procedures.

RESULTS

The experimental results reported here are in chronological order. Following the results of the distribution of copper, manganese and zinc in brain tissue, it was intended to apply ultracentrifugation, gel chromatography, enzyme hydrolysis, neutron activation analysis, *in vivo* and *in vitro* labelling techniques for the identification of soluble copper, manganese, and zinc proteins in brain tissue.

3.1. Distribution of Copper, Manganese and Zinc in Brain Tissue[97]

The concentrations of copper, manganese and zinc were determined in 11 different regions of a calf brain (Table V). The outstanding feature of these results is the high concentration of all three trace elements in the pineal body.

The pineal body was investigated in more detail (Table VI), and the concentrations of copper, manganese and zinc were compared with those found in the pons, the latter serving as an internal standard. In all four pineal bodies the concentrations of copper, manganese and zinc were exceedingly high. Further analysis were carried out on cow and pig brains (Table VII and VIII). Again the pineal body showed high concentrations of trace metals.

The distribution of copper, manganese and zinc in human brains was studied (Table IX and X). In this case, the concentrations of copper, manganese and zinc in the pineal body are only slightly above the "average". By comparison of the results on calf and cow brains, a con-

siderable decrease in the concentrations of three elements were observed. Both the calves and the pig were approximately 3 months old whereas the cows and human subjects were over 2 and 70 years of age respectively.

The data in Table X indicate that the highest copper content was found in locus caeruleus. The results are in good agreement with published data[35].

3.2. Extraction of Soluble Proteins

The experimental procedure was already described on page 25. The percentage of copper, manganese and zinc which was found extractable from 9 different regions of brain tissue is given in Table XI. This fraction contains the soluble metallo-proteins and also whatever other forms and quantities of these three trace elements are extractable under the particular experimental conditions.

Table XI indicates that the relative amounts of copper, manganese and zinc which can be extracted from homogenized tissue of different regions of the brain are essentially constant (40%) except the pineal body. About 70-80% of trace metals in the pineal body was found in the soluble fraction.

3.3. Evidence for Metallo-Proteins of Copper, Manganese and Zinc

Basal ganglia extract was fractionated on a Bio-Gel P-6 column (1.8 x 30 cm) and the metal content in the eluant was determined by neutron activation analysis. The results of the analysis for soluble metallo-proteins in basal ganglia are shown in Fig. I. The metal concentration of the eluant is plotted against eluant weight in a block

TABLE V

Distribution of Copper, Manganese and Zinc in a Calf Brain

Region	Cu	Mn	Zn
		(μg/g dry wt.)	
Pineal body	19	2.9	95
Pons	5.9 ± 0.1	1.3 ± 0.1	30 ± 1
Medulla	5.9	1.2	39
Mid-brain	6.2 ± 0.1	1.2 ± 0.1	36 ± 1
Spinal cord	3.7	1.1	16
Basal ganglia	13	1.7	70
Cerebellum, white matter	3.3 ± 0.8	1.2 ± 0.3	19 ± 4
Cerebellum, grey matter	8.2	2.9	51
Cerebral cortex, white matter	4.8 ± 0.7	0.7 ± 0.1	20 ± 2
Cerebral cortex, grey matter	7.0 ± 1.8	1.3 ± 0.3	66 ± 10
Corpora quadrigemina	7.2 ± 0.1	1.6 ± 0.1	39 ± 1

Values for which errors are quoted indicate duplicate or triplicate analysis. The error reflects the maximum spread of the respective results. In order to obtain an approximate value for the content of trace metals/g wet weight, the data should be divided by 5.

TABLE VI

Copper, Manganese and Zinc in the Pineal Body and Pons of Calf Brains

	Pineal body			Pons		
	Cu	Mn	Zn	Cu	Mn	Zn
	(μg/g dry wt.)			(μg/g dry wt.)		
Brain 1	19	2.9	95	5.9	1.3	30
Brain 2	25	3.2	112	7.5	1.3	33
Brain 3	21	2.9	80	7.3	1.0	32
Brain 4	42	3.3	102	11	1.2	32

TABLE VII

Distribution of Copper, Manganese and Zinc in Cow Brains

Region	Cu (μg/g dry wt.)		Mn (μg/g dry wt.)		Zn (μg/g dry wt.)	
	Brain 1	Brain 2	Brain 1	Brain 2	Brain 1	Brain 2
Pineal body	8.6	7.8	2.2	2.2	87	93
Pons	2.7 ± 0.5	4.2 ± 0.3	0.6 ± 0.1	0.7 ± 0.1	16 ± 1	24 ± 1
Medulla	4.0		0.9		27	
Mid-brain	4.4		1.2		22	
Basal ganglia	8.9 ± 0.7	11 ± 2	1.4 ± 0.1	1.6 ± 0.1	52 ± 3	64 ± 3
Cerebrall cortex, white matter	2.5		0.6		21	
Corpora quadrigemina	4.0		1.0		21	

TABLE VIII

Distribution of Copper, Manganese and Zinc in a Pig Brain

	Cu	Mn	Zn
		(μg/g dry wt.)	
Pineal body	22	4.2	72
Pons	6.9 ± 1.1	0.7 ± 0.1	17 ± 3
Medulla	12	1.0	18
Mid-brain	11	1.2	26
Basal ganglia	19 ± 1	1.5 ± 0.2	49 ± 2
Corpora quadrigemina	9.8	0.8	21

TABLE IX

Distribution of Copper, Manganese and Zinc in Human Brain

	Brain 1		
	Cu	Mn (μg/g dry wt.)	Zn
Pineal body	8.5	1.0	63
Right occipital white matter	5.3	0.85	21
Right pre-frontal white matter	6.6	0.78	17
Floor of the fourth ventricle	8.5	0.80	19
Thalamus	11	1.2	35
Right caudate	23	2.6	57

TABLE X

Distribution of Copper, Manganese and Zinc in Human Brain

	Brain 2		
	Cu	Mn (μg/g dry wt.)	Zn
Left locus caeruleus	49	1.6	31
Right locus caeruleus	59	1.7	27
Left cortex	11	0.68	14
Right cortex	12	0.71	18
Left thalamus	13	1.7	33
Right thalamus	14	1.8	33
Left caudate	26	2.6	54
Right caudate	25	2.5	49

TABLE XI

Extraction of Trace Metals into

0.15 M CH_3COONH_4/0.01 M EDTA

	% extracted		
	Cu	Mn	Zn
Pineal body	68	78	80
Basal ganglia	36	35	38
Cerebellum, grey	36	47	55
Cerebellum, white	43	41	69
Cortex, grey	36	41	40
Cortex, white	47	48	50
Midbrain	39	47	62
Pons	27	44	57
Corpora quadrigemina	38	44	22

Fig. I

Evidence for the Soluble Metallo-Proteins of Copper, Manganese and Zinc in Brain Tissue

Fig. Ia: Copper

Fig. Ib: Manganese

Fig. Ic: Zinc

Bio-Gel P-6 column (1.8 x 30 cm)

P = Protein region

I = Ionic region

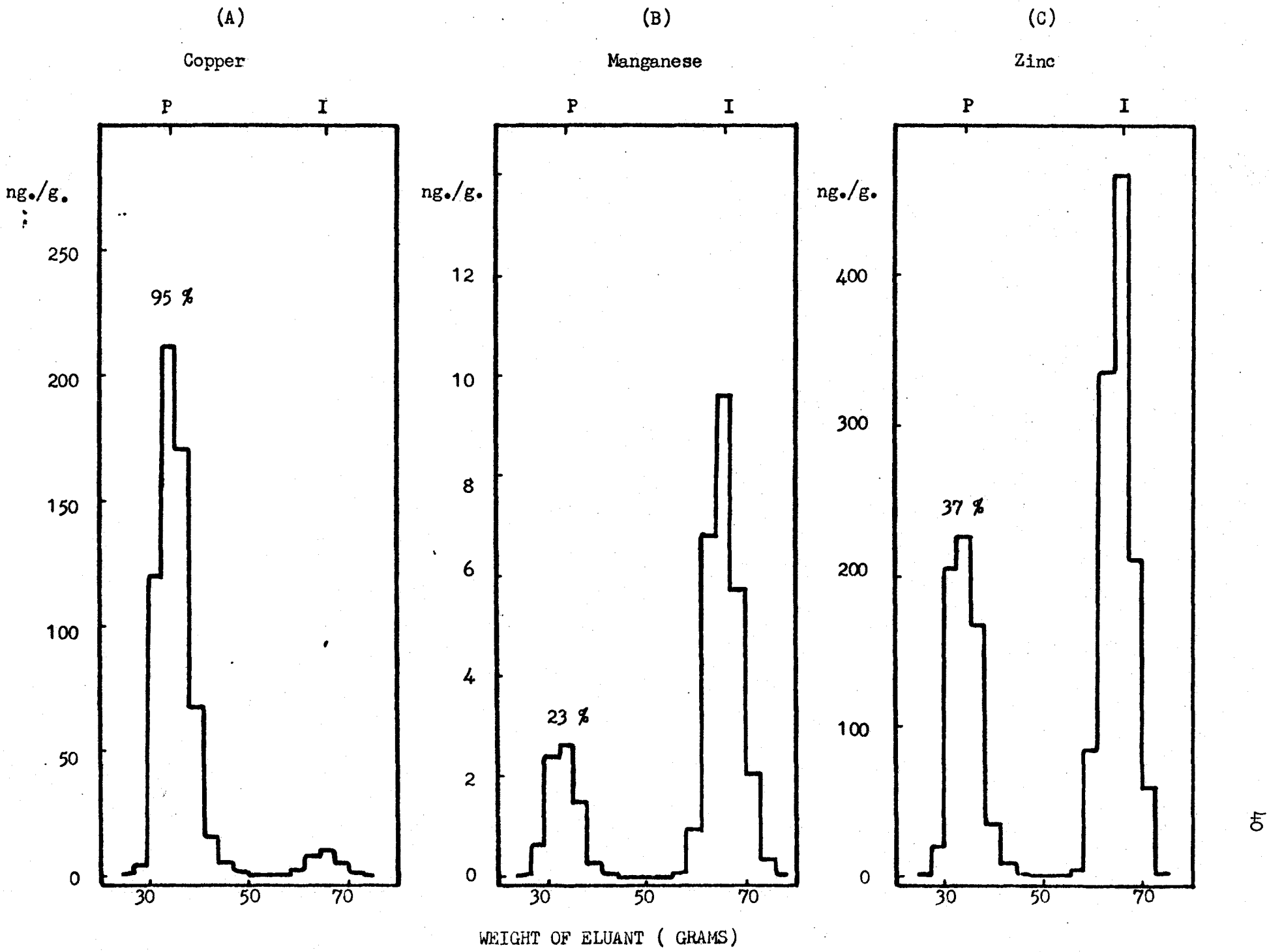

(A)
Copper
P
I
ng./g.
250
200
150
100
50
0
95 %
30
50
70
(B)
Manganese
P
I
ng./g.
12
10
8
6
4
2
0
23 %
30
50
70
(C)
Zinc
P
I
ng./g.
400
300
200
100
0
37 %
30
50
70
WEIGHT OF ELUANT (GRAMS)

diagram. The possible presence of metallo-proteins is revealed by the appearance of peaks within the protein region of the diagram. Fig. I shows the presence of copper, manganese and zinc proteins in the basal ganglia extract. About 95% copper, 23% manganese, and 37% zinc in the extract were present in the protein region.

3.4. In Vitro Labelling Experiments

3.4.1. Manganese-54 and zinc-65 labelling

The brain tissue was homogenized with about one-half to one-third its weight of the extraction solution containing carrier-free ^{54}Mn or ^{65}Zn at 4°C. After being centrifuged at 100,000 x g for one hour the brain extract was fractionated on a Bio-Gel P-100 column (1.8 x 37.5 cm). The distribution of manganese-54 and zinc-65 activities are shown in Fig. II and III respectively.

3.4.2. Copper-64 labelling

The experimental procedure was the same as discussed above for manganese-54 and zinc-65.

The in vitro labelling of basal ganglia extract with "carrier-free" copper-64 in the presence of EDTA leads to the formation of protein complexes. A typical result is shown in Fig. IV. Another four runs gave the same result.

In order to show that the copper-64 was indeed protein bound the system was studied carefully by enzyme hydrolysis. To a separate portion of the "spiked" basal ganglia extract, a few milligrams of pronase was

Fig. II

In Vitro Labelling with Manganese-54 in the Soluble Fraction of Basal Ganglia

Bio-Gel P-100 column (1.8 x 37.5 cm)

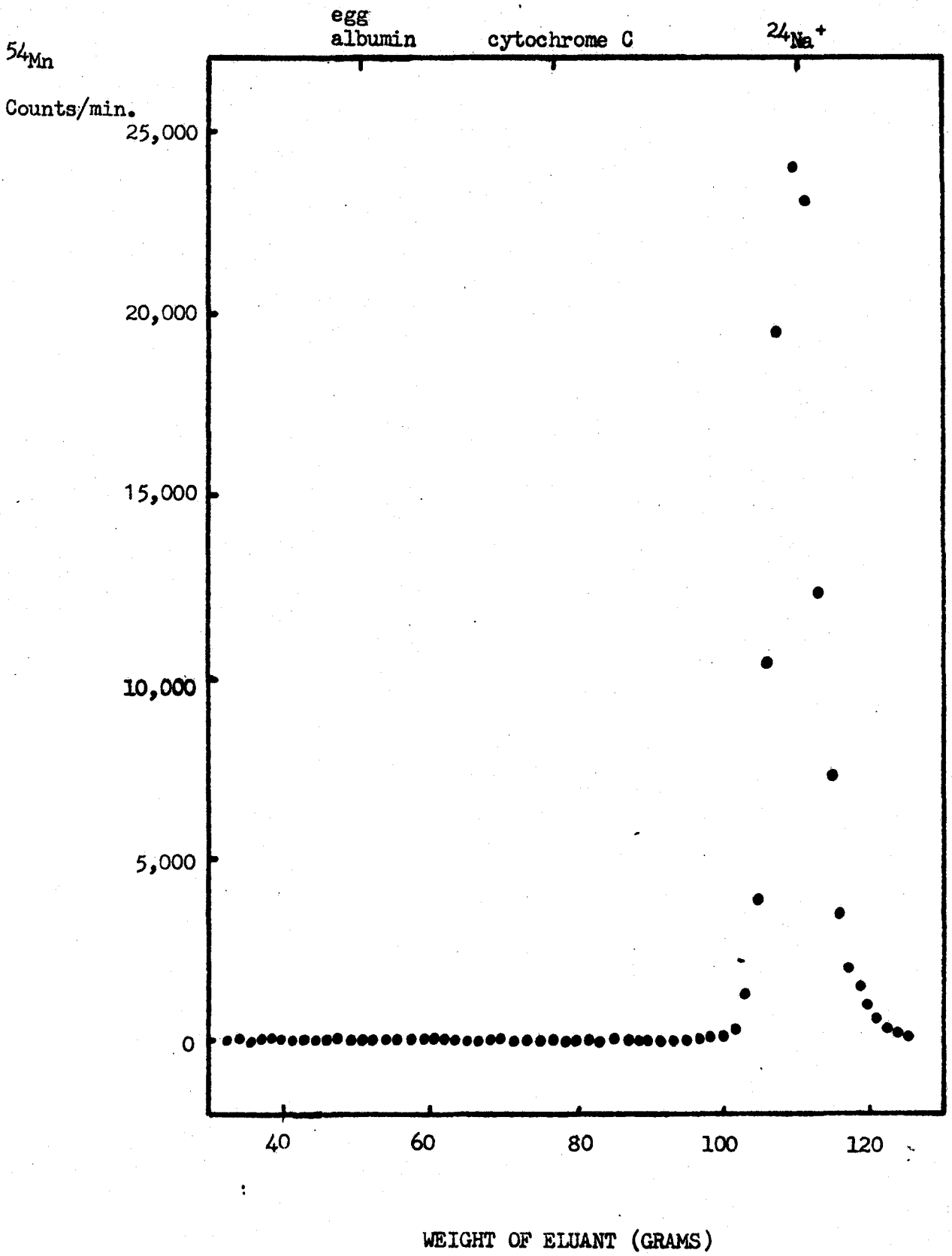

egg
albumin
cytochrome C
$^{24}Na^{+}$
^{54}Mn
Counts/min.
25,000
20,000
15,000
10,000
5,000
0
40
60
80
100
120
WEIGHT OF ELUANT (GRAMS)

Fig. III

In Vitro Labelling with Zinc-65 in the Soluble Fraction of Basal Ganglia

Bio-Gel P-100 column (1.8 x 37.5 cm)

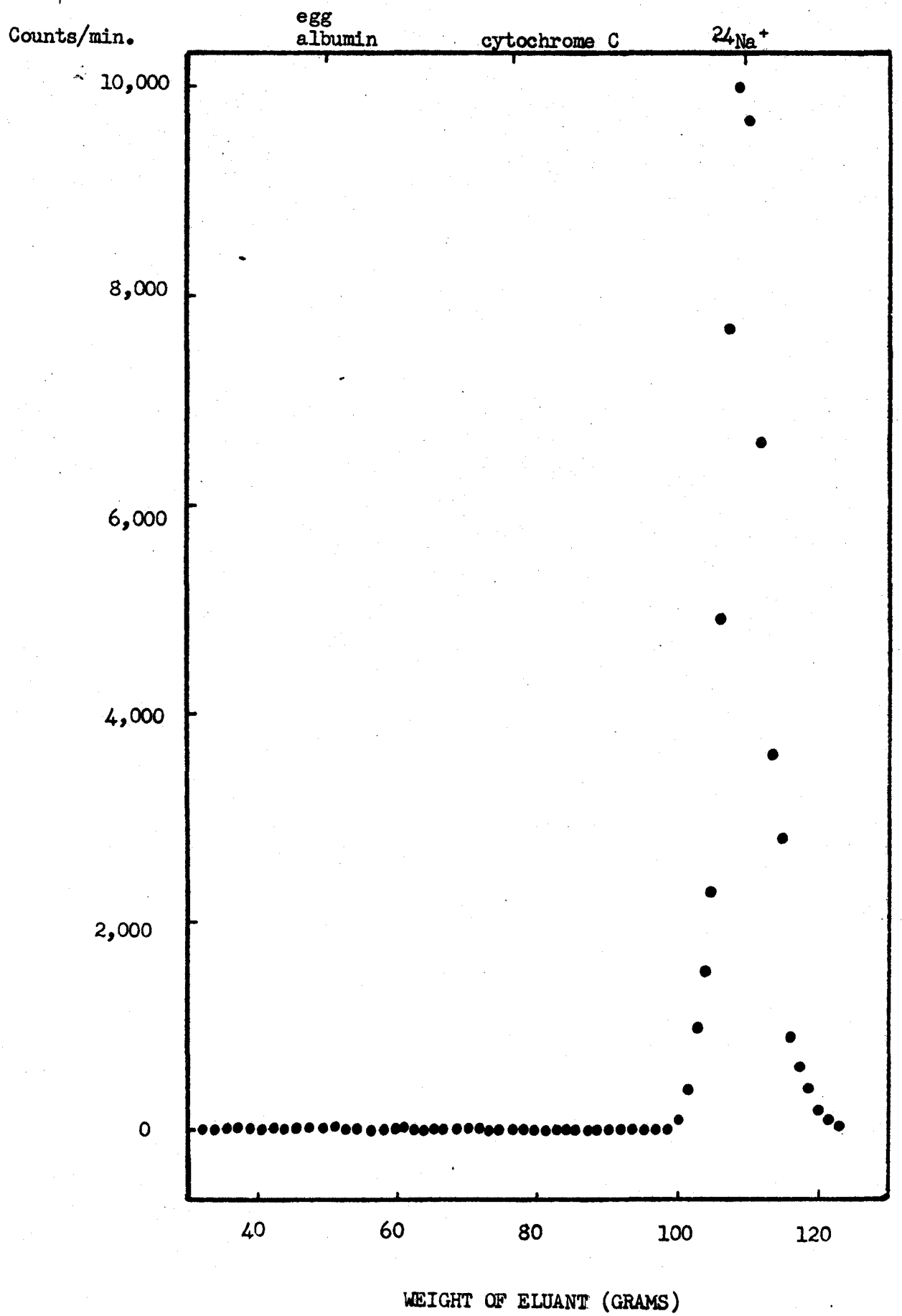
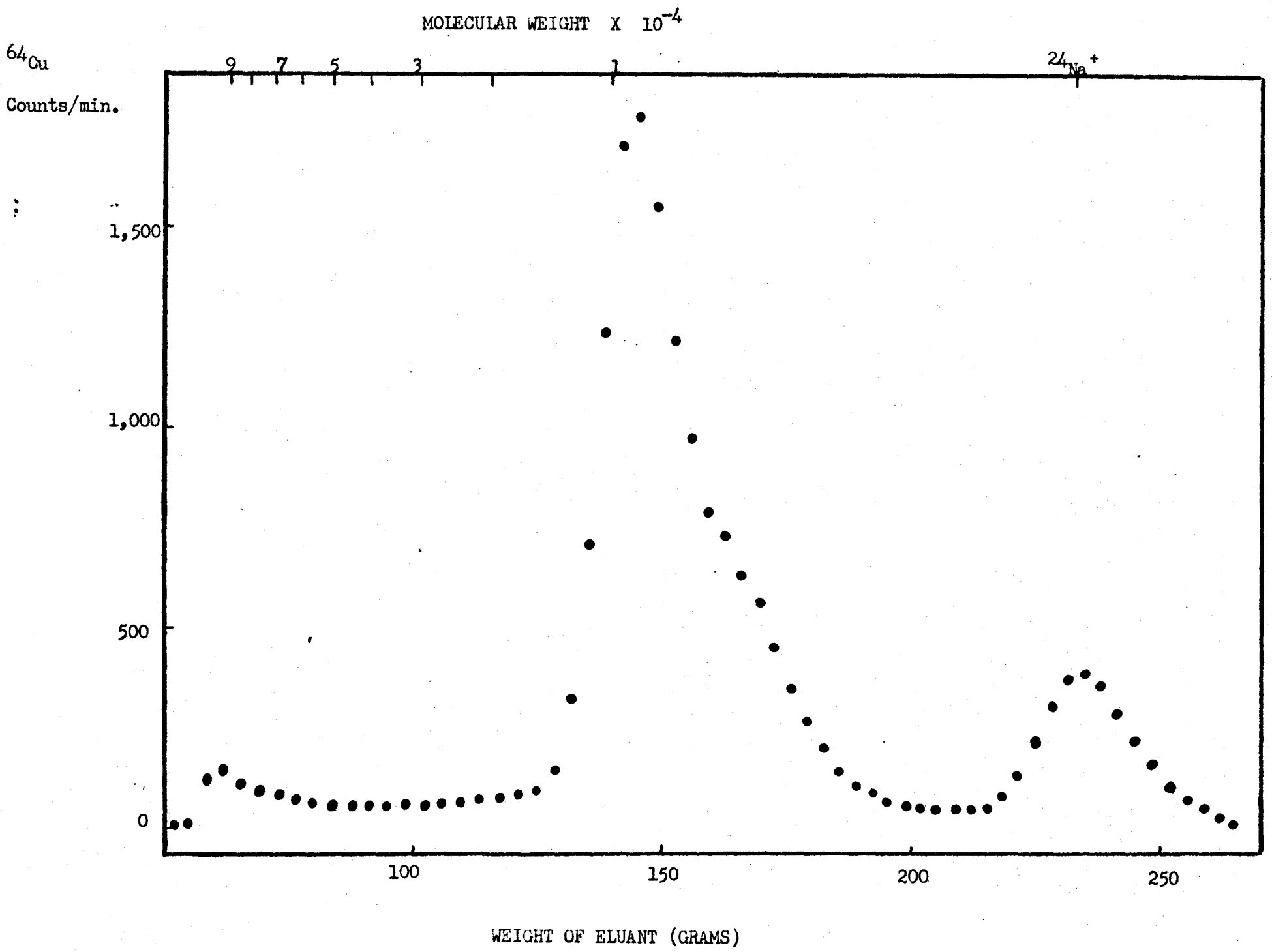

^{65}Zn
Counts/min.
egg albumin
cytochrome C
$^{24}Na^{+}$
10,000
8,000
6,000
4,000
2,000
0
40
60
80
100
120
WEIGHT OF ELUANT (GRAMS)

Fig. IV

In Vitro Labelling with Copper-64 in the Soluble Fraction of Basal Ganglia

Bio-Gel P-100 column (2.6 x 44 cm)


MOLECULAR WEIGHT X 10^{-4}
9
7
5
3
1
24_{Na}^{+}
64_{Cu}
Counts/min.
1,500
1,000
500
0
100
150
200
250
WEIGHT OF ELUANT (GRAMS)

added. The enzyme hydrolysis took place at 40°C for 28 hours. The hydrolyzed basal ganglia extract was chromatographed on the same Bio-Gel P-100 column as used above. The result is shown in Fig. V which represents one of three identical experiments. All copper-64 activity appeared always in the "ionic" fraction.

3.5. In Vivo Labelling Experiment

0.8 millicurie of "carrier-free" manganese-54 in 1 ml saline solution was injected into a rat weighing 250 grams. A six day circulating time was allowed to ensure that enough manganese-54 passed through the blood-brain barrier. After this time the rat was sacrificed by cardiac puncture and the brain was stored in a freezer at -20°C. The methods of extraction of soluble proteins and gel chromatographic fractionation were described early. About 7% of total manganese-54 activity was found in the protein region corresponding to a molecular weight of ~ 60,000. The results are shown in Fig. VI.

In order to show that the injected manganese-54 was indeed protein bound the system was studied carefully by enzyme hydrolysis. To 0.3 ml brain extract, 3 mg pronase was added. The enzyme hydrolysis took place at 40°C for 23 hours. Both the non-hydrolyzed and hydrolyzed brain extracts were chromatographed on a Bio-Gel P-10 column (1.6 x 18.5 cm). About 7% of the total manganese-54 activity was found in the protein region of the non-hydrolyzed brain extract. Only 4% of the total manganese-54 activity was found in the protein region of the hydrolyzed brain extract. The results are shown in Fig. VII.

Fig. V

In Vitro Labelling of Copper-64 in the Soluble Fraction of Basal Ganglia after Enzyme Hydrolysis Bio-Gel P-100 column (2.6 x 44 cm)

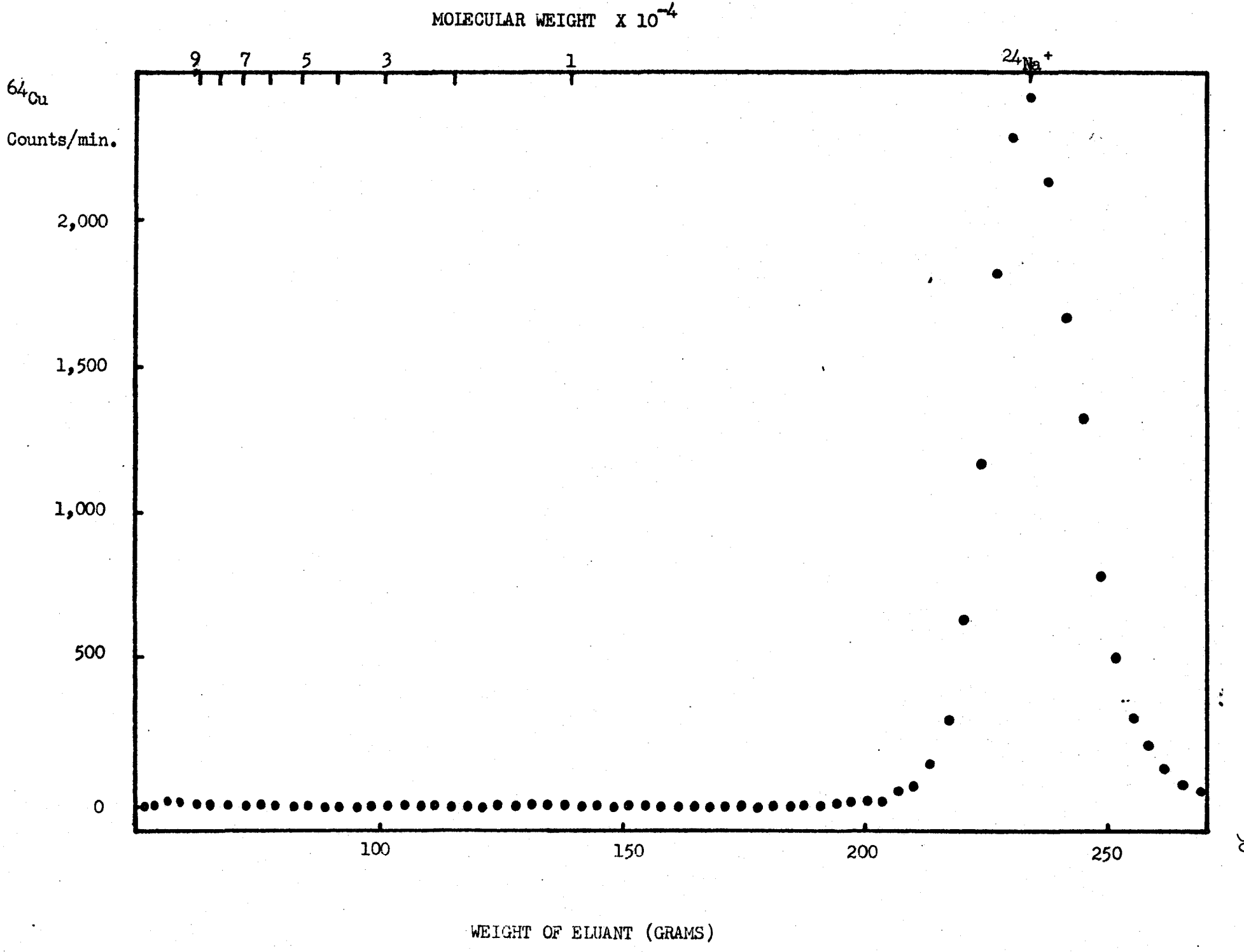

MOLECULAR WEIGHT X 10^{-4}
9
7
5
3
1
24_{Na^+}
64_{Cu}
Counts/min.
2,000
1,500
1,000
500
0
100
150
200
250
50
WEIGHT OF ELUANT (GRAMS)

Fig. VI

In Vivo Labelling of Manganese-54 in Brain Tissue

Bio-Gel P-100 column (2.6 x 44 cm)

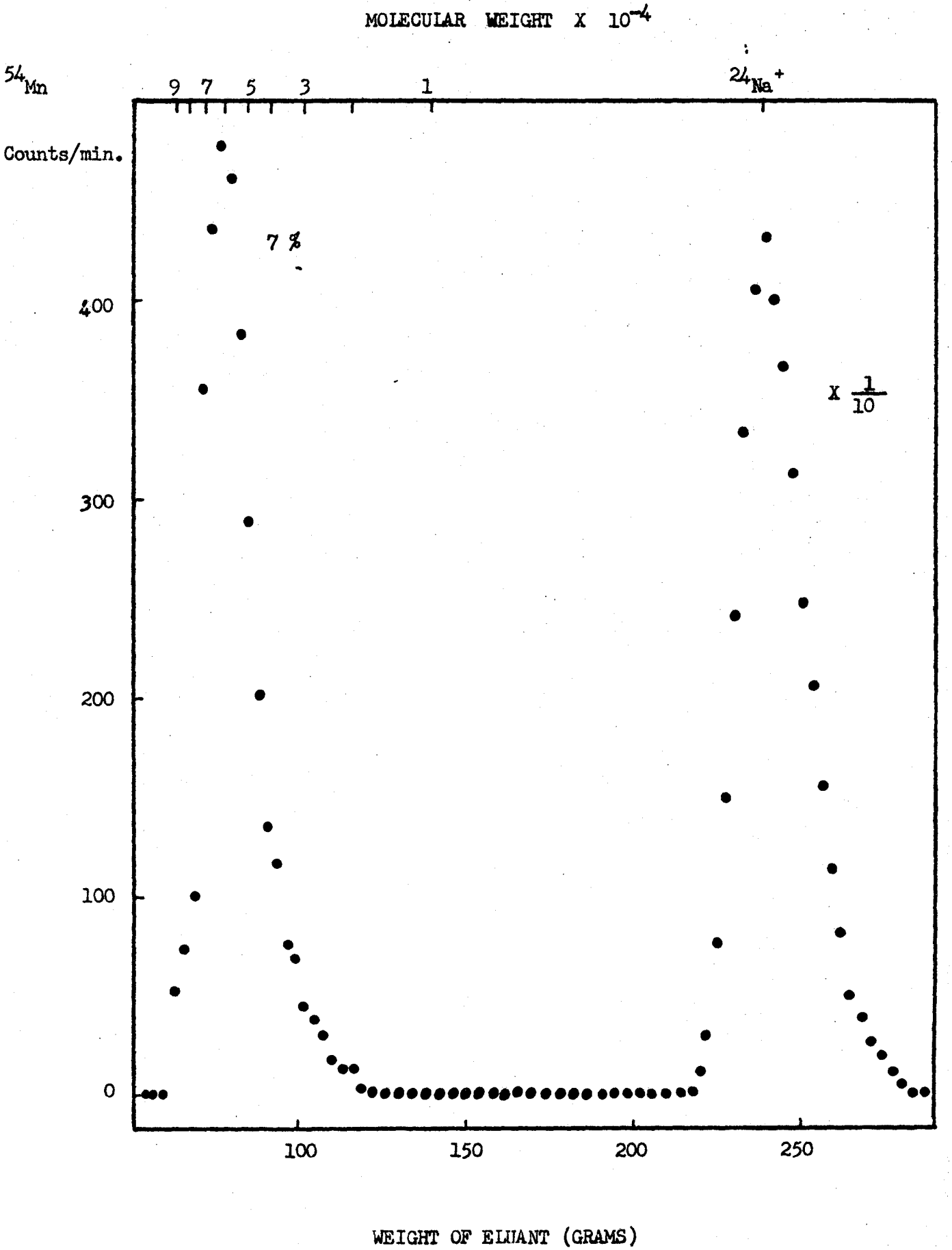

MOLECULAR WEIGHT X 10^{-4}
^{54}Mn
9 7 5 3 1
$^{24}Na^{+}$
Counts/min.
7 %
400
300
200
100
0
X $\frac{1}{10}$
100 150 200 250
WEIGHT OF ELUANT (GRAMS)

Fig. VII

In Vivo Labelling of Manganese-54 in Brain Tissue

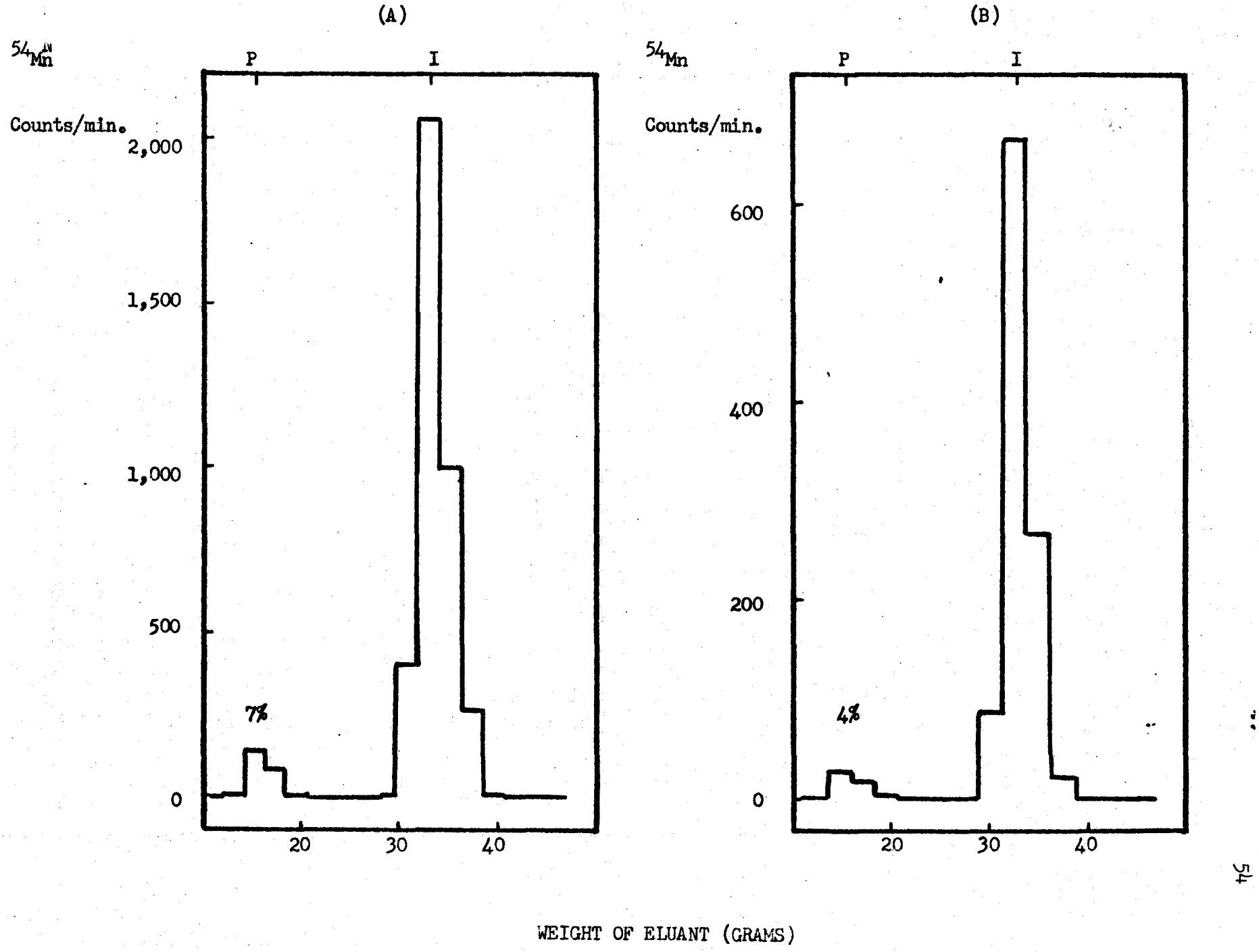
Fig. VIIa: Before Enzyme Hydrolysis

Fig. VIIb: After Enzyme Hydrolysis

Bio-Gel P-10 column (1.6 x 18.5 cm)

P = Protein region

I = Ionic region


(A)
(B)
^{54}Mn
^{54}Mn
P
I
P
I
Counts/min.
Counts/min.
2,000
1,500
1,000
500
0
600
400
200
0
7%
4%
20
30
40
20
30
40
WEIGHT OF ELUANT (GRAMS)

3.6. Identification of Soluble Metallo-Proteins in Basal Ganglia

9.5 grams basal ganglia was homogenized with 4.5 grams solution, 0.15 M in ammonium acetate and 0.01 M in EDTA (pH = 6.9). 1.2 grams of the resulting extract was chromatographed on a Bio-Gel P-100 column (2.6 x 47.5 cm). The details of extraction of soluble proteins, gel chromatography and metal analysis have been described in the experimental part. Immediately after the chromatographic separation the column was calibrated using blue dextrin, human transferrin, serum albumin, egg albumin, cytochrome C and $^{24}Na^{+}$. The molecular weight calibration curve of the column is shown in Fig. VIII.

The results of the analysis for soluble metallo-proteins in basal ganglia are shown in Fig. IX. The metal concentration of the eluant is plotted against the eluant weight. The presence of a metallo-protein is revealed by the appearance of a peak within the protein region of the diagram. This graph (Fig. IX) shows the presence of copper proteins of apparent molecular weights of > 90,000 (~15%), ~35,000 (~18%), and ~10,000 (~57%), a manganese protein of a molecular weight ~61,000 (~20%) and zinc proteins of molecular weights of ~87,000 (~8%), and ~34,000 (~12%).

In order to prove that the three trace elements are indeed protein bound the system was studied further by the combination of enzyme hydrolysis, gel chromatography and neutron activation analysis. The experimental procedure is different from those mentioned earlier. The basal ganglia extract was obtained by homogenization of the tissue with

Fig. VIII

Molecular Weight Calibration of Bio-Gel P-100

(2.6 x 47.5 cm)

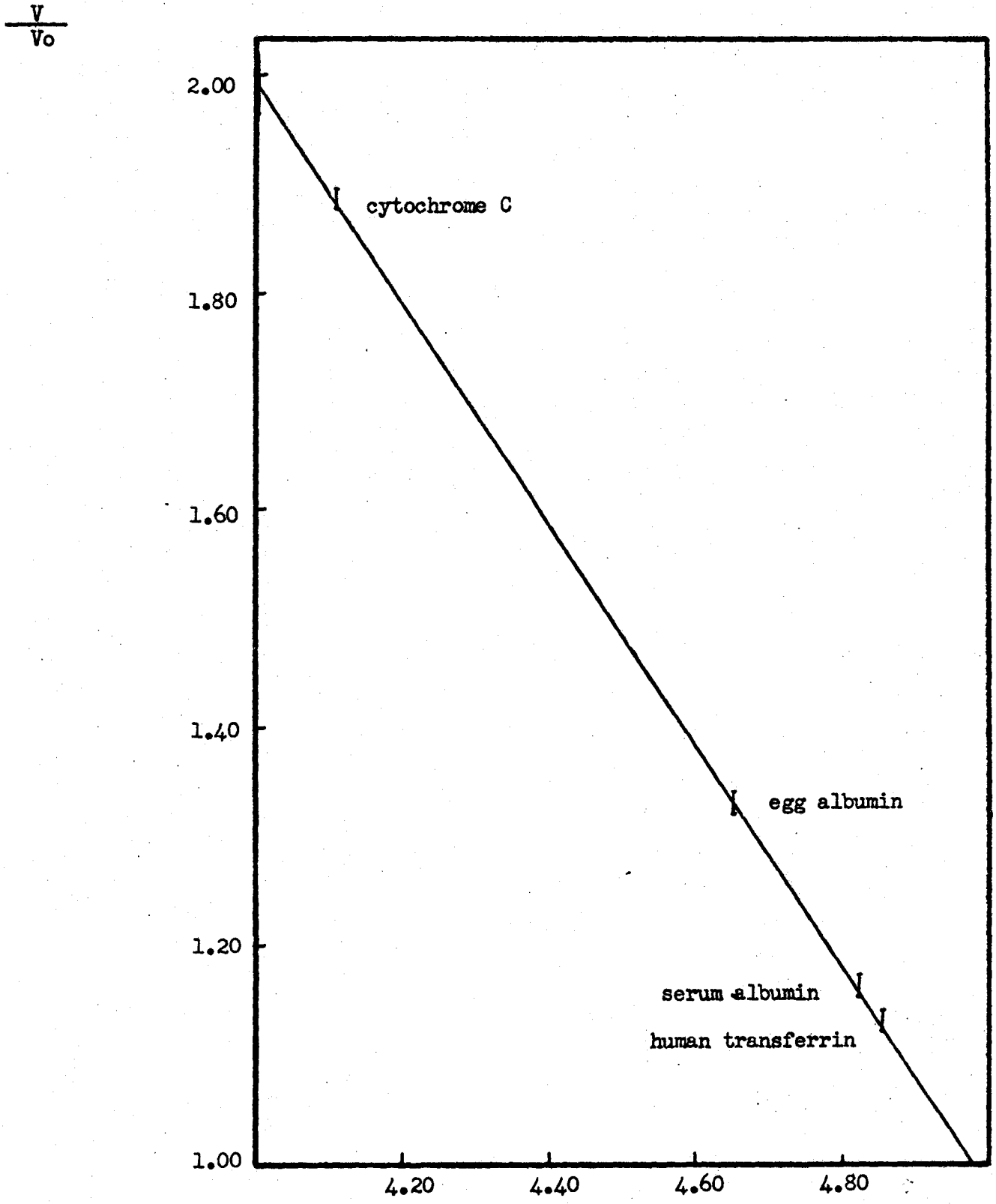
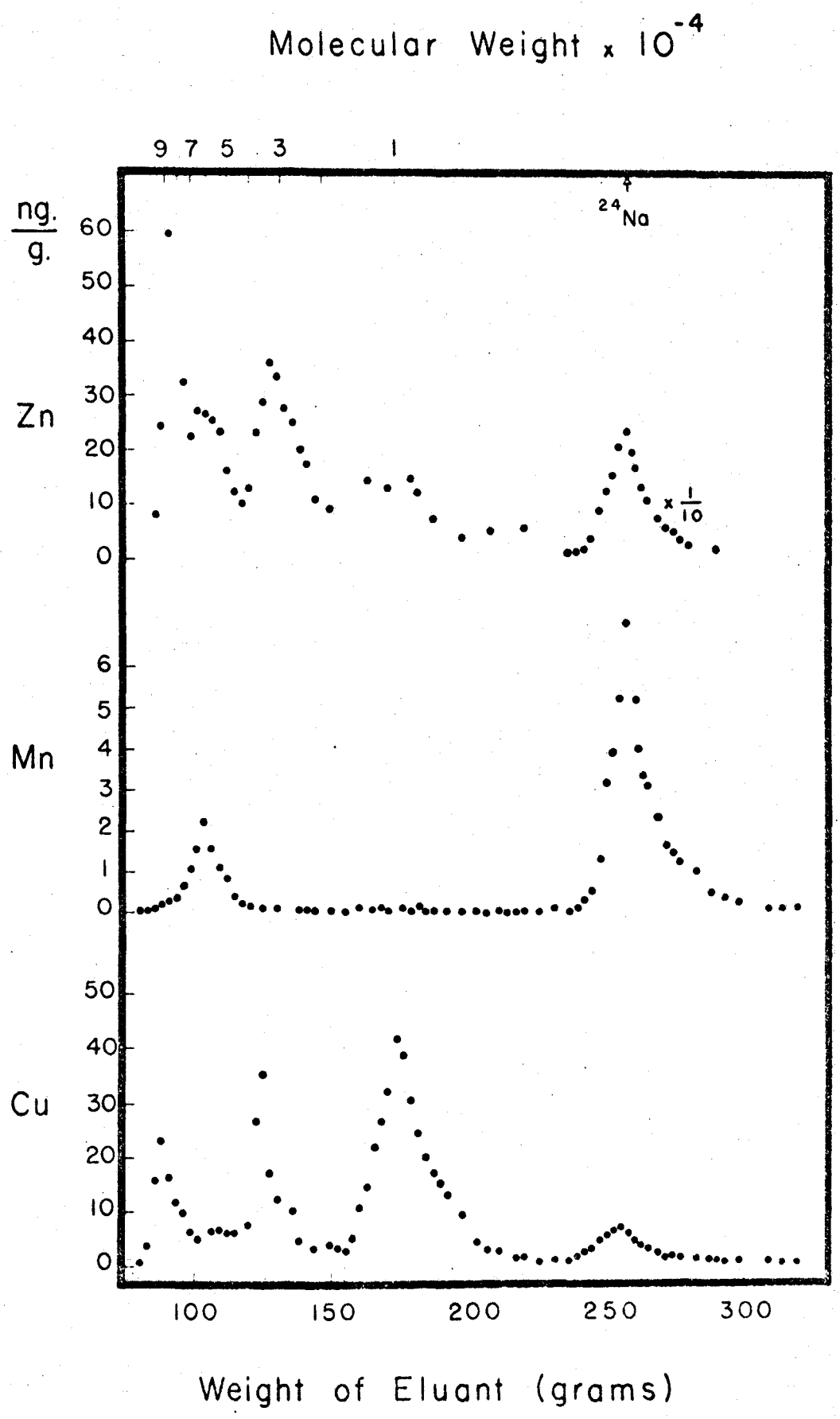

V/Vo
2.00
1.80
1.60
1.40
1.20
1.00
cytochrome C
egg albumin
serum albumin
human transferrin
4.20
4.40
4.60
4.80
LOGARITHM OF MOLECULAR WEIGHT

Fig. IX

Soluble Metallo-Proteins of Copper, Manganese and Zinc in Basal Ganglia

Bio-Gel P-100 column (2.6 x 47.5 cm)


Molecular Weight x 10^{-4}
9 7 5 3 1
ng./g.
Zn
Mn
Cu
^{24}Na
x 1/10
60
50
40
30
20
10
0
6
5
4
3
2
1
0
50
40
30
20
10
0
100
150
200
250
300
Weight of Eluant (grams)

a solution 0.15 M in ammonium acetate (pH = 6.9). One-half of it was chromatographed on a Bio-Gel P-6 column (1.8 x 30 cm). To the remaining extract, about 4 mg pronase was added. The enzyme hydrolysis took place at 40°C for 44 hours. At the end of the hydrolysis the supernatant was adjusted to 0.01 M in EDTA and was let stand for 1 hour at room temperature before fractionation through the same Bio-Gel P-6 column. The details of the metal analysis have been described on page 27. The results are shown in Fig. X, XI and XII. In these figures only the protein regions are shown. Fig. Xa, XIa and XIIa show the results in the absence of enzyme hydrolysis. After enzyme hydrolysis, only 12% of copper (Fig. Xb), 60% of manganese (Fig. XIb), and 8% of zinc (Fig. XIIb) are left in the protein region.

3.7. Identification of Soluble Metallo-Proteins in the Pineal Body

Calves' brains were obtained from Essex Packers, Hamilton, and stored in the freezer at -20°C. 35 pineal bodies (~4.8 grams) were homogenized with 2.5 grams solution 0.15 M in ammonium acetate and 0.01 M in EDTA (pH = 6.9). 2.2 grams of the resulting extract was chromatographed on a Bio-Gel P-100 column (2.6 x 44 cm). The details of extraction of soluble proteins, gel chromatography and metal analysis have been described in the experimental part. Immediately after the chromatographic separation the column was calibrated using blue dextrin, human transferrin, bovin albumin, egg albumin, cytochrome C and $^{24}Na^{+}$. The molecular weight calibration curve of the column is shown in Fig. XIII.

Fig. X

Copper Proteins in Basal Ganglia (Elution on a Bio-Gel P-6 Column)

Fig. Xa: Before Enzyme Hydrolysis

Fig. Xb: After Enzyme Hydrolysis

Bio-Gel P-6 column (1.8 x 30 cm)

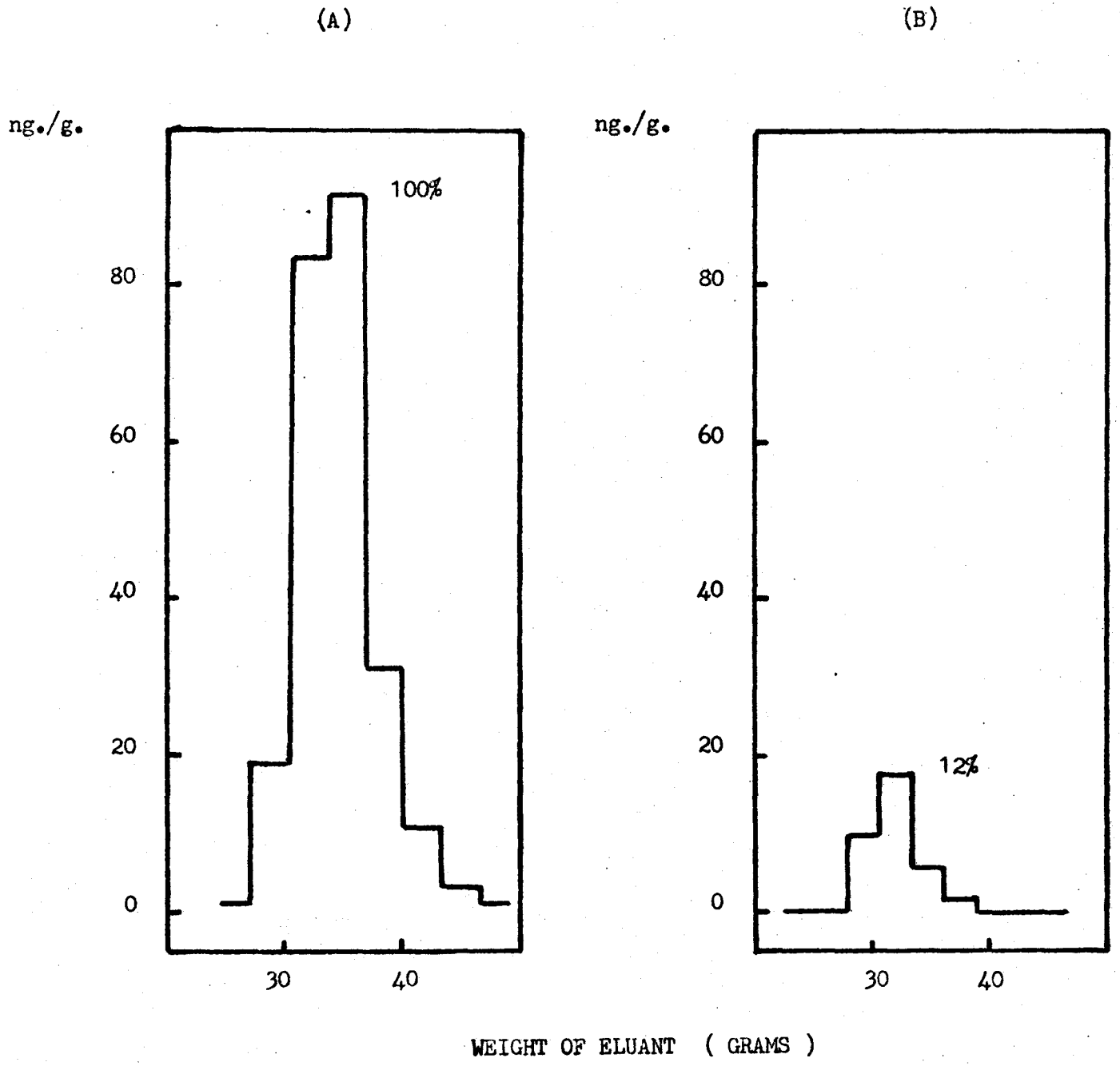

(A)
(B)
ng./g.
ng./g.
100%
12%
80
60
40
20
0
30
40
WEIGHT OF ELUANT (GRAMS)

Fig. XI

Manganese Proteins in Basal Ganglia (Elution on a Bio-Gel P-6 Column)

Fig. XIa: Before Enzyme Hydrolysis

Fig. XIb: After Enzyme Hydrolysis

Bio-Gel P-6 column (1.8 x 30 cm)

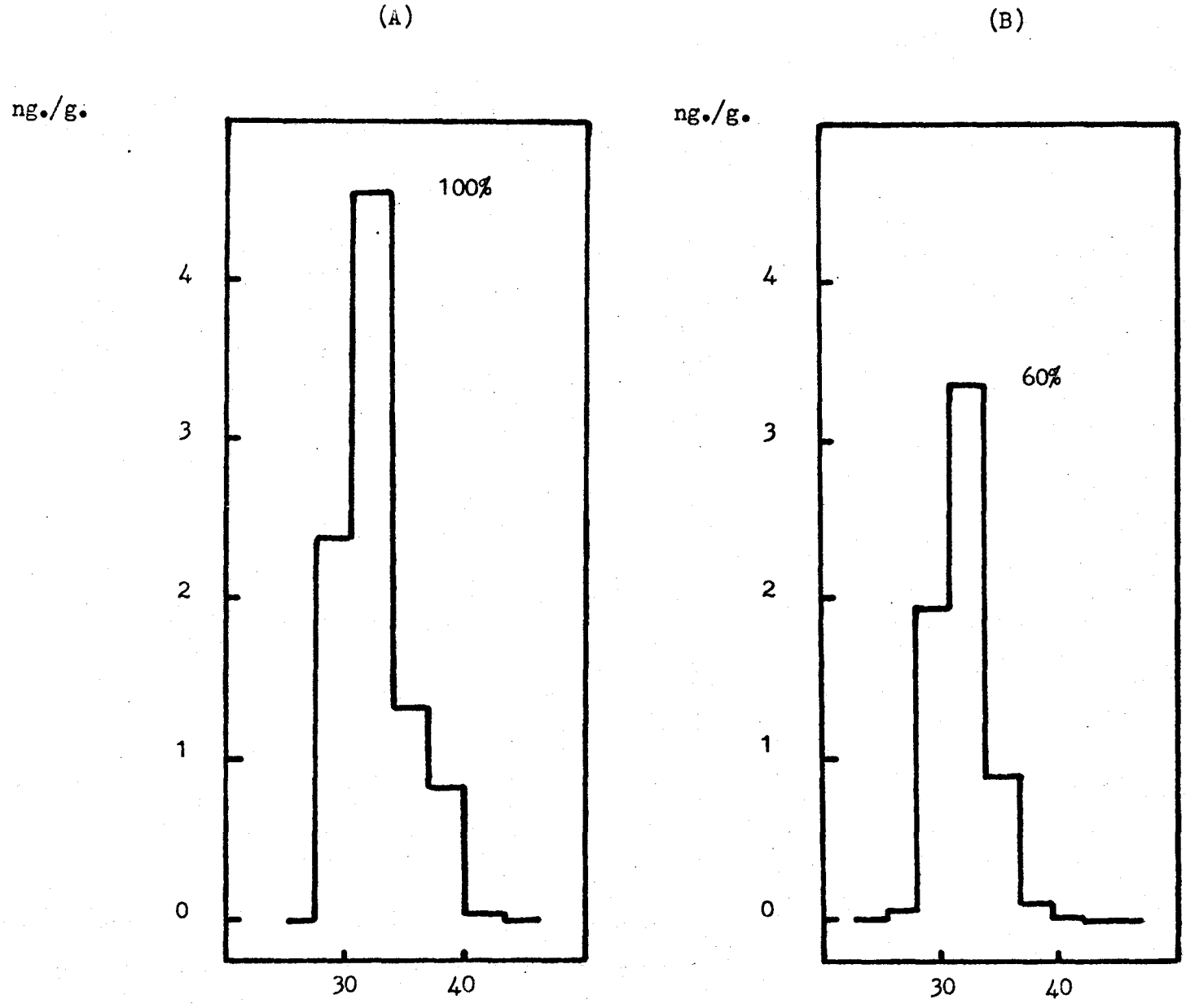

(A)
(B)
ng./g.
ng./g.
100%
60%
4
3
2
1
0
30
40
WEIGHT OF ELUANT (GRAMS)

Fig. XII

Zinc Proteins in Basal Ganglia (Elution on a Bio-Gel P-6 Column)

Fig. XIIa: Before Enzyme Hydrolysis

Fig. XIIb: After Enzyme Hydrolysis

Bio-Gel P-6 column (1.8 x 30 cm)

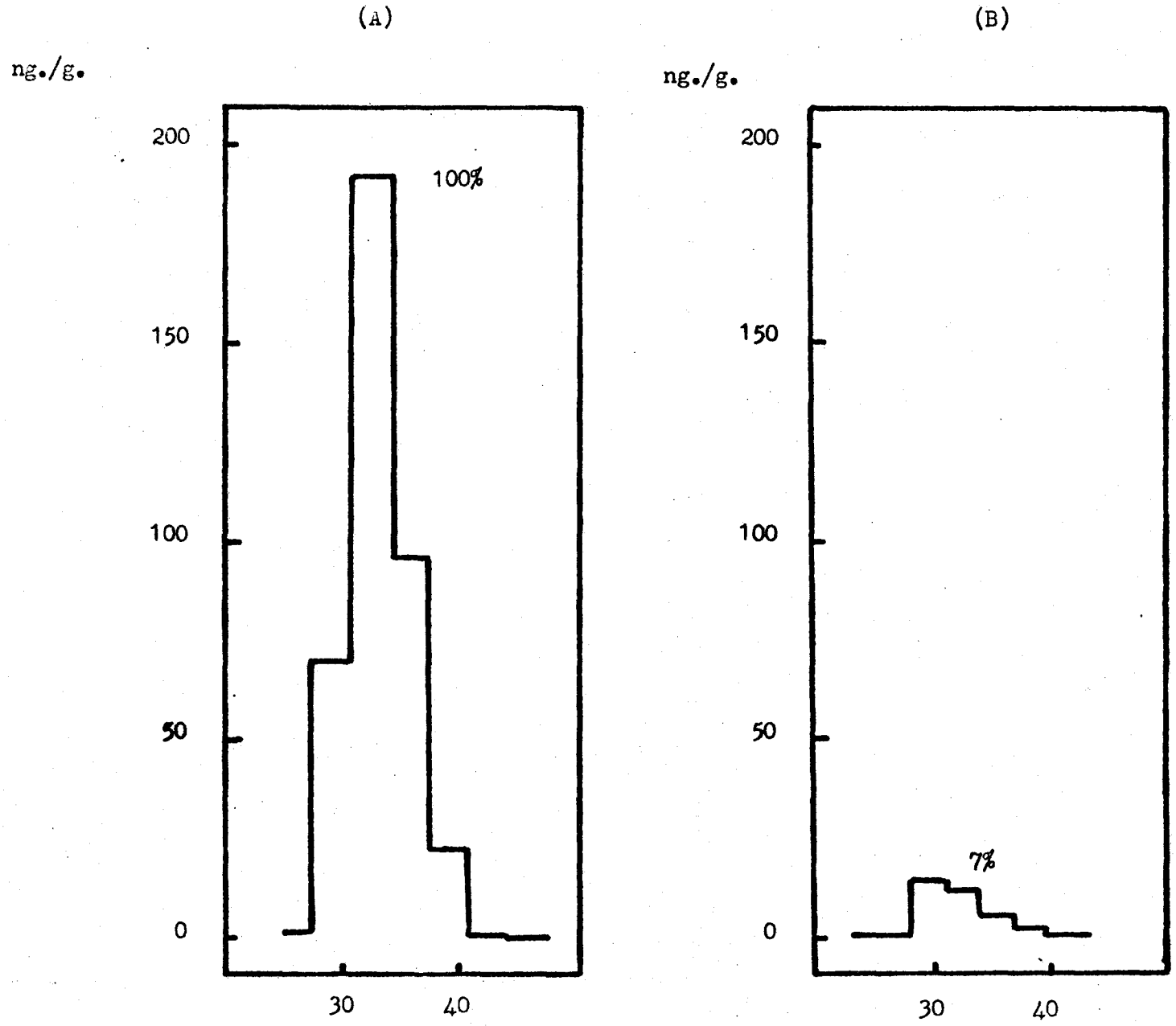
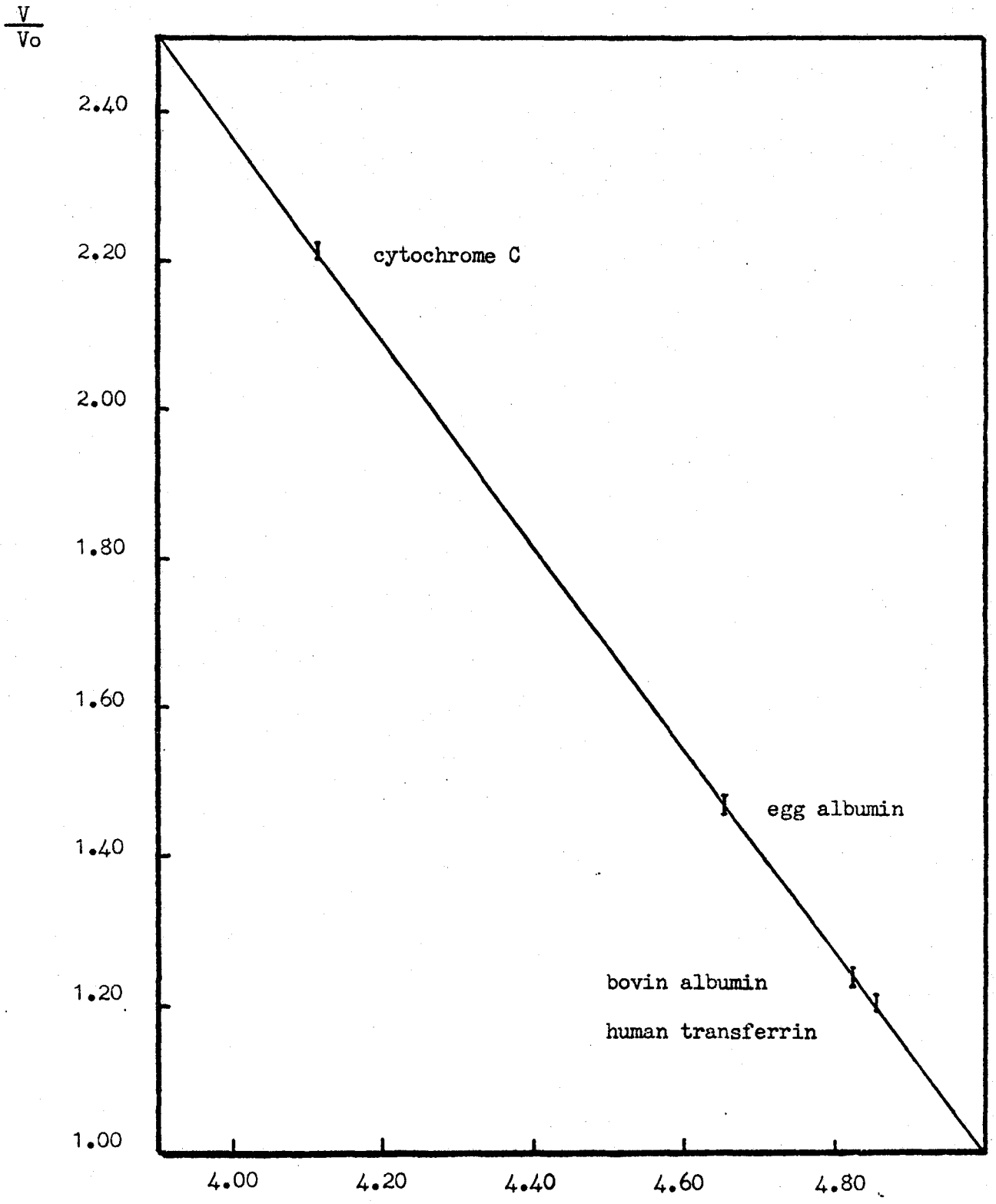

(A)
ng./g.
200
150
100
50
0
100%
30
40
(B)
ng./g.
200
150
100
50
0
7%
30
40
WEIGHT OF ELUANT (GRAMS)

Fig. XIII

Molecular Weight Calibration of Bio-Gel P-100

Column (2.6 x 44 cm)

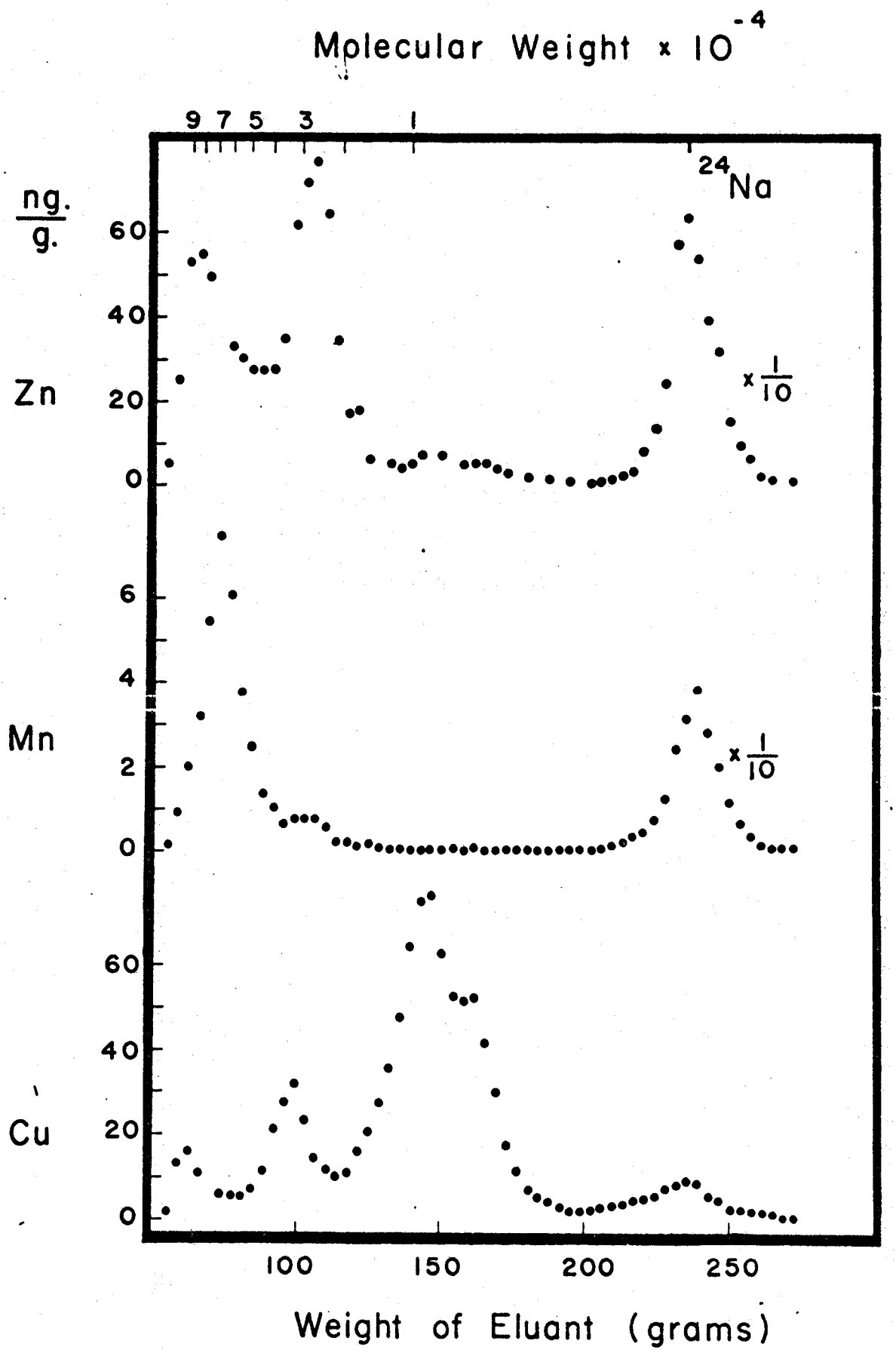

V/Vo
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
cytochrome C
egg albumin
bovin albumin
human transferrin
4.00
4.20
4.40
4.60
4.80
LOGARITHM OF MOLECULAR WEIGHT

The results of the analysis for soluble metallo-proteins in the pineal body are shown in Fig. XIV. The metal concentration of the eluant is plotted against eluant weight. The presence of a metallo-protein is revealed by the appearance of peak within the protein region of the diagram. This graph (Fig. XIV) shows the presence of copper proteins of apparent molecular weights of > 90,000 (~5%), ~31,000 (~16%) and ~9,000 (~72%); a manganese protein of molecular weight ~62,000 (~15%) and zinc proteins of molecular weights of ~87,000 (~7%) and ~28,000 (~9%).

Fig. XIV

Soluble Metallo-Proteins of Copper, Manganese and Zinc in the Pineal Body

Bio-Gel P-100 column (2.6 x 44 cm)


Molecular Weight x 10^-4
9 7 5 3 1
24Na
ng./g.
Zn
60
40
20
0
x 1/10
Mn
6
4
2
0
x 1/10
Cu
60
40
20
0
100
150
200
250
Weight of Eluant (grams)

DISCUSSION

4.1. Distribution of Copper, Manganese and Zinc in Brain Tissue[97]

Neutron activation analysis was used for the simultaneous determination of copper, manganese and zinc in the brains of calves, cows, pigs and human. Measurements of these three elements in as many as 11 different regions of the brain showed that the highest content is always found in the pineal body of young mammals.

On the basis of the results in Table V to X, the concentrations of the trace metals may be related to age. The concentrations of copper, manganese and zinc are high especially in young mammals. The most pronounced part is the pineal body in which the concentrations of trace metals decrease as a function of age. The pineal body was investigated in more detail (Table VI), and the concentrations of copper, manganese and zinc were compared with those found in the pons, the latter serving as an internal standard. It is quite obvious from all the published analysis and my own data, that considerable fluctuations in content of trace metals are found even within one species. The analysis of pons is assumed to give an indication of overall levels. The concentrations of copper in brain no. 4 provide the justification for this approach. In all four pineal bodies the concentrations of copper, manganese and zinc were exceedingly high. Further analyses were carried out on cow, pig and human brains (Tables VII, VIII and IX). Again, the pineal bodies showed high concentrations of trace metals. Typical values for calf

brains are: copper, ~ 20 μg/g; manganese, ~ 3 μg/g; and zinc, ~ 90 μg/g; for cow brains are: copper, ~ 8 μg/g; manganese, ~ 2 μg/g; zinc, ~ 90 μg/g; for the pig brain are: Cu, ~ 22 μg/g; manganese, ~ 4 μg/g; and zinc ~ 70 μg/g; for the human brain are: copper, ~ 8 μg/g; manganese, ~ 2 μg/g; and zinc, ~ 60 μg/g dry pineal tissue. The calves were approximately 3 months old whereas the cows and the human subjects were over 2 and 70 years of age respectively.

So far, only manganese has been reported to be extremely high in the pineal of rabbits (15 μg/g dry wt.)[51]. The results were 5 times higher than those reported in this work. However, the precision of the results were questionable (26 and 3.3 μg/g dry wt. respectively).

The pineal body is now known to be a neuro-endocrine transducer - that is, its cells take a stimulus such as light and convert it to an hormonal production by activating the sex glands, thyroid and pituitary[98]. There are only a few reports which deal with quantitative changes in the pineal body as a function of age. Hellman and Carlsson[99] reported that there is a decrease with age in oxygen consumption, glucose oxidation and conversion of labelled glucose to labelled amino acids in the pineal body of the goat. Krass and Le Bella[100] also found that in the total glucose oxidation, both C-1 and C-6, and C-1/C-6 ratios were higher in pineal bodies from young (5-10 months) than from adult (3-8 years) bovines. Zweens[101] reported a decrease in the phospholipid content with age in the pineal body of rat. Thus copper, manganese and zinc may be related to these biochemical changes as a function of age.

Apart from the pineal body, the concentrations of copper, manganese and zinc are also high in basal ganglia (for calf brains: copper,

~ 13 μg/g; manganese, ~ 1.7 μg/g; and zinc, ~ 70 μg/g). This part of the brain seems to accumulate trace metals and is severely affected in chronic manganese poisoning. Generally, the grey matter contained higher concentrations of trace metals than white matter. This may be due to higher protein concentration in the grey matter.

The distribution of trace metals in old human subjects is different from young mammals. The highest content exists in the caudate nucleus which is a part of basal ganglia. Typical values are: copper, ~ 25 μg/g; manganese, ~ 2.5 μg/g; zinc, ~ 50 μg/g dry tissue. However, the highest copper content was found in the locus caeruleus. The results are in good agreement with published data[35]. Thompson[35] explained that presumably this unusual high concentration of copper in this area was related to the pigmentation. This pigmentation only occurs in old mammals.

For almost all brain regions as a first approximation, the ratio of copper : manganese : zinc was roughly constant. In most publications results of several determinations are usually presented as averages which tend to obscure this effect.

4.2. Extraction of Soluble Proteins

Table XI indicates that the relative amounts of copper, manganese and zinc which can be extracted from homogenized tissue of different regions of the brain are essentially constant (~ 40%). The only exception is the pineal body in which more than two-thirds of the trace metals is in the soluble fraction. The earlier work (Tables V and VI)

had shown that this part of the brain contains the largest total content of the three trace elements. It is now clear that this high concentration is mainly due to an increase of the metal amounts in the soluble fraction.

According to a review of the literature by Lajtha[102], most aqueous media extract between 30 to 50 per cent of total brain proteins. In the determination of the intracellular distribution of copper in brain, Porter and Ainsworth[36] found that about 47% of copper was in the cortex supernatant. Cuzner and co-workers[103] used 0.32 M sucrose containing 1 mM EDTA (pH = 7.4) for the extraction of soluble proteins. Because of the prevention of contamination, the solution for extraction of soluble brain proteins was 0.15 M in ammonium acetate and 0.01 M in EDTA (pH = 6.9). Stored in a polyethylene bottle, this extracting solution was found to contain negligible amounts of copper, manganese and zinc. From the analytical point of view the presence of 0.01 M EDTA served important purposes. Firstly, it provides a standard for the distinction of metallo-proteins and metal-protein complexes. The metal-EDTA complexes of the three elements provide the base line for the distinction of these two species since the stability constants of metal-EDTA complexes of Cu^{2+}, Mn^{2+} and Zn^{2+} are 18.7, 13.5 and 16.4 respectively. Secondly, it breaks down any polymerization products of metal-hydroxides which could appear in gel-chromatography experiments as high molecular weight species. Finally, it prevents the contamination of the protein fractions before gel chromatographic fractionation. A detailed discussion on this point will be given on page 77.

4.3. Evidence for Metallo-Proteins of Copper, Manganese and Zinc

It is evident from Fig. Ia that the majority of copper (~ 95%) in the soluble fraction of basal ganglia occurs as one or several metallo-proteins with molecular weights greater than 6,000. It also indicates that the stability constants of these metallo-proteins are greater than that of the Cu-EDTA complex (log K = 18.7). Porter and Floch[37] found a copper protein - cerebrocuprein - in the soluble fraction of brain tissue. Carrico and Deutsch[39] indicated that this copper protein is stable upon dialysis against 0.01 M EDTA. Therefore at least part of the copper content in the protein region of Fig. Ia is due to cerebrocuprein. Only 5% occurs in the "ionic" region and is presumably due to the Cu-EDTA complex. This indicates that this element exists in the brain tissue to a very small extent in weak complexes or possibly even as the free ion.

Fig. Ib shows that 23% of the total manganese occurs in the protein region with a molecular weight greater than 6,000. This implies the presence of at least one manganese protein in the basal ganglia supernatant. This manganese protein is stable in the presence of 0.01 M EDTA. So far, no manganese proteins have been reported in brain tissue. The majority of the manganese occurs in the "ionic" region of the elution curve which shows that this portion exists in the original brain tissue as either weak complexes or the free ion.

Fig. Ic shows that 37% of the total zinc occurs in the protein region with a molecular weight greater than 6,000. This indicates that

one, or more than one, zinc protein are present in the basal ganglia supernatant. Several zinc enzymes have been found in the soluble fraction of brain tissue and they are listed in Table IV. These zinc enzymes may contribute to the protein peak in Fig. Ic. The stabilities of these proteins in the presence of 0.01 M EDTA will be discussed later. The majority of the zinc content (~ 63%) is in the "ionic" region.

4.4. In Vitro Labelling Experiments

4.4.1. Manganese-54 and Zinc-65 labelling

Fig. II and III show the results of manganese-54 and zinc-65 *in vitro* labelling experiments. No sign of manganese and zinc activities were found in the protein region indicating that both manganese and zinc did not form any protein complexes in the presence of EDTA. All the added manganese-54 and zinc-65 appeared in the ionic fraction in a form of metal-EDTA complexes. It seems that no results were obtained from these experiments. However, several valuable conclusions can be obtained.

It is evident that by the combination of neutron activation analysis and gel chromatography metallo-proteins of manganese and zinc were shown to be present in brain supernatant (Fig. Ib and Ic). The presence of several zinc enzymes in the soluble fraction of brain tissue has already been reported[73, 76, 82, 68]. On the other hand, no manganese and zinc proteins were found in the *in vitro* experiments. This indicates that the soluble metallo-proteins of manganese and zinc in brain tissue are formed only *in vivo* but not *in vitro*, which in turn, might imply that the

metallo-proteins are physiologically important. The metals in the metallo-proteins are firmly bound so that no exchange between radioactive and inactive metals takes place in vitro under the experimental conditions.

Separate experiments have shown that metal-protein complexes of manganese and zinc were formed in vitro in the absence of EDTA. On the other hand, no metal-protein complexes were found in the presence of EDTA. This means that EDTA can be used to distinguish between metallo-proteins and weak metal-protein complexes.

Both Fig. II and III show that the addition of "carrier-free" manganese-54 and zinc-65 to the brain supernatant finally ended up in the "ionic" fraction. This indicates that any possible external or internal contaminations to the protein region will be impossible. In other words, if any contaminants of manganese and zinc are introduced in any operation before the start of the gel-chromatographic elution, they will eventually appear only in the "ionic" fraction.

4.4.2. Copper-64 labelling

Fig. IV shows the results of in vitro labelling of basal ganglia extract with "carrier-free" copper-64. Less than 1% of the total copper-64 activity is bound to a protein of a molecular weight greater than 90,000. The majority of the copper-64 activity (~ 80%) is associated with one or more than one proteins of molecular weight equal to 9,000-10,000. A shoulder was found on the lower molecular weight side of the peak which is obviously not "Gaussian-shaped". This indicates that the

majority of the copper-64 activity is probably associated with two proteins. About 20% of the copper-64 activity was found in the "ionic" fraction.

It is evident from Fig. V that the copper-64 activity disappeared in the protein region after enzyme hydrolysis. This means that the copper-64 bound macromolecules, formed in vitro, are metallo-proteins, because only proteins are hydrolyzed by pronase.

The results of these in vitro experiments also agree with that of the gel chromatography-activation analysis experiment (Fig. I). This indicates that copper proteins must be present in the soluble fraction of brain tissue and they may be physiologically important.

The in vitro formation of copper proteins in the presence of EDTA indicates that the copper atoms are firmly bound to the proteins and the stability constants of these metallo-proteins are greater than that of the copper-EDTA complex. The appearance of copper-64 activity in the protein region may be due to a surplus of the complex forming protein in tissue. A rapid exchange of the originally bound copper with copper-64 is another possibility.

Porter and Folch[37] found a copper protein with a molecular weight of 35,000 - cerebrocuprein - in the soluble fraction of brain tissue. On the other hand, no sign of copper-64 activity was found in the protein of a molecular weight between 30,000 and 40,000 indicating that there is no exchange between radioactive copper and the inactive copper in cerebrocuprein.

4.5. In Vivo Labelling Experiment

The incorporation of radiomanganese in blood has been studied extensively by Foradori et al.[104], Bertinchamps and Cotzias[54], Evans[105] and Hancock[53], but no one has attempted to incorporate radiomanganese in brain tissue. There was evidence to show the presence of at least one manganese protein in basal ganglia extract by gel chromatography-activation analysis (Fig. I). However, no manganese-54 activity was found in the protein region by the in vitro experiment (Fig. II). In order to search further for manganese proteins, radiomanganese was incorporated to the brain tissue by in vivo labelling techniques.

It is evident from Fig. VI that the in vivo manganese-54 labelling into rat brain leads to the formation of a manganese protein of a molecular weight of ~ 60,000. A separate experiment showed that this manganese protein decomposed upon disc gel electrophoresis.

In order to show that the manganese-54 was indeed protein bound the system was studied carefully by enzyme hydrolysis. The results were shown in Fig. VII.

The distribution of manganese-54 activity in the non-hydrolyzed brain extract is shown in Fig. VIIa. The result showed that ~ 7% of the total manganese-54 activity was present in the protein region. This agrees with the result given in Fig. VI.

The distribution of manganese-54 activity in brain extract after enzyme hydrolysis is shown in Fig. VIIb. Only 4% of total manganese-54 activity was found in the protein fraction and the rest was in the ionic

fraction. This indicates that more than one-third of the manganese protein was hydrolyzed by the addition of pronase. The remaining two-thirds of the manganese-54 activity is probably due to incomplete hydrolysis or due to the hydrolyzed fragments with molecular size larger than the exclusion limit of the Bio-Gel P-10 column.

Based on the results of the gel chromatography-activation analysis experiment (Fig. I), in vitro and in vivo experiments (Fig. II and VII), it is obvious that there is definitely a soluble manganese protein in the brain tissue. The metal to protein binding is stronger than that of the metal-EDTA complex. Further investigation of this manganese protein involving studies of the distribution of natural occurring isotopes in vivo in different areas of brain tissue will be discussed in the later sections.

4.6. Identification of Soluble Metallo-Proteins in Basal Ganglia

4.6.1. Soluble copper proteins

It is evident from Fig. IX that the majority of the copper (~ 57%) in the soluble fraction of basal ganglia occurs as a metallo-protein with a molecular weight of 10,000. This metallo-protein is apparently also formed by the in vitro addition of copper-64 to tissue extract (Fig. IV) which could indicate that there is a surplus of the complex forming protein in the tissue. A rapid exchange of the originally bound copper with copper-64 is another possibility. No decision can be made on the basis of the available data. Previous workers had indications of this copper protein in brain tissue. Porter and Ainsworth[38] observed a

copper protein of "a molecular weight of the order of only 10,000" during their work on the isolation of cerebrocuprein. Carrico and Deutsch[39] also comment on the fact that "most of the copper in brain extracts was associated with an acidic yellow protein which was well separated from the cerebrocuprein by the first DEAE-cellulose chromatographic step". Uricase - a copper protein with a molecular weight of 12,000 was found in mammalian liver[106]. However, it is absent in the soluble fraction of tissue and has not been reported in brain tissue. The in vitro copper-64 labelling of liver and kidney in the presence of EDTA did not show the formation of such a complex. No such copper protein has been found in serum, erythrocytes and leukocytes. All the evidence given so far appears to indicate that this copper protein of molecular weight ~ 10,000 is specific for brain. Furthermore, if one examines this part of the elution curve on Fig. IV and IX one sees that there is a shoulder on the low molecular weight side and the peak is not "Gaussian-shaped". This indicates that there are in fact at least two copper proteins in this region. Detailed discussion will be seen in the next section.

The second largest peak accounting for 18% of the total copper occurs in an elution position corresponding to a molecular weight of ~ 35,000. It must be cerebrocuprein discovered by Porter and Floch[37]. It contains two gram atoms of copper per mole of protein. The copper is strongly bound and only relatively small amounts of it could be removed from the protein by dialysis against EDTA and 1, 10 phenanthroline under various conditions[66]. Cerebrocuprein can retain all of its copper on

dialysis at a pH as low as 3.9. On the basis of the given data, one can calculate that about 70 μg of cerebrocuprein contributed to the copper peak in Fig. IX. This indicates that the method used here is sensitive and only a small amount of tissue is enough for the identification of metallo-proteins. As mentioned earlier this protein was recently shown to be identical with heptacuprein in liver and erythrocuprein in erythrocytes, and all three have consequently renamed as cytocuprein[39].

The third peak (~ 15% of total copper, molecular weight > 90,000) in the elution curve is difficult to interpret. Though copper enzymes such as cytochrome oxidase (molecular weight ~ 93,000), monoamine oxidase (molecular weight ~ 170,000) and dopamine β-hydroxylase (molecular weight ~ 290,000) have been reported in brain tissue[40, 41, 43], they are present in the mitochondrial fraction only, and therefore unlikely to contribute to this peak. The best explanation at present is that most if not all of this peak is due to ceruloplasmin (molecular weight ~ 151,000, containing 8 gram atoms of copper per mole of protein) from blood serum. From the amount of copper in the particular elution position and other data the original 9.5 grams basal ganglia had to contain between 0.3 and 0.5 ml of blood which appears to be a reasonable figure. Furthermore, Friede[107] indicated that cereloplasmin is also involved in brain metabolism.

In order to show that the copper in basal ganglia was indeed protein bound the system was studied by enzyme hydrolysis. The results are shown in Fig. X representing only the protein regions. Fig. Xa shows the result in the absence of enzyme hydrolysis. Fig. Xb shows that only 12% of the

copper is left in the protein region after enzyme hydrolysis. The remaining 12% of the copper is probably due to the following: (1) the incomplete hydrolysis of certain copper proteins; (2) the hydrolyzed fragments with molecular size larger than the exclusion limit of the Bio-Gel P-6 columns.

4.6.2. Soluble manganese protein

The gel chromatography-activation analysis experiment shows the presence of one manganese protein with a molecular weight of 61,000 in the soluble fraction of basal ganglia (Fig. IX). This manganese protein accounts for ~ 20% of total manganese in the extract. It is not formed *in vitro* (Fig. II). The *in vivo* manganese-54 labelling experiment provides the same results (Fig. VI). About 7% of the total manganese-54 activity was found in the *in vivo* experiment, because only six day circulating time was allowed. The time might not be enough for the actual physiological equilibrium. So far no manganese protein has been found in brain tissue. Hancock[53] has identified a serum manganese protein with a molecular weight of 70,000, which is a β_1-globulin. This serum manganese protein, formed both *in vitro* and *in vivo*, is similar to both in electrophoretic and chromatographic behaviour to the iron-binding protein, transferrin. On the other hand, the soluble manganese protein in brain tissue is not formed *in vitro* and is unstable upon disc gel electrophoresis. In fact, these two manganese proteins are quite different on the basis of their molecular weights, *in vitro* labelling and electrophoretic properties. At this point, it must be emphasized that the contribution

of manganese from serum is negligible because the content of manganese in serum is in the order of ppb. Whether the soluble manganese protein is specific for brain tissue remains to be seen. In the absence of EDTA, a weak complex with a molecular weight of ~ 45,000 is formed *in vitro*. In each experiment comparatively large quantities of manganese appear in the "ionic" fraction. This indicates that this element exists in the brain tissue to some extent in very weak complexes or possibly even as the free ion.

In order to show that the manganese in basal ganglia was indeed protein bound the system was studied by the combination of enzyme hydrolysis, gel chromatography and neutron activation analysis. The experimental procedures have been described on page 60. The results are shown in Fig. XI. Fig. XIa shows the result in the absence of enzyme hydrolysis. Fig. XIb shows that about 60% of the manganese is left in the protein region after enzyme hydrolysis for 44 hours. A separate experiment has indicated that only 50% of the manganese is left in the protein region after pronase hydrolysis for 72 hours. This agrees with the *in vivo* manganese-54 labelling experiment (Fig. VI) in which two-thirds of the manganese were left in the protein region after enzyme hydrolysis. The appearance of manganese in the protein region is probably due to, (1) the incomplete hydrolysis of the manganese protein (molecular weight ~ 61,000); (2) the hydrolyzed fragments with molecular size larger than the exclusion limit of the Bio-Gel P-6 column.

4.6.3. Soluble zinc proteins

The distribution of zinc in the soluble fraction of basal ganglis is shown in Fig. IX. By the comparison of copper and manganese, the zinc diagram looks very complicated.

Most of the protein bound zinc (~ 12% of total zinc) appears in the elution position corresponding to a molecular weight of ~ 34,000. However, the peak shape - particularly after comparison with the copper peak in the same region - indicates that more than one zinc protein occurs here. This peak can be satisfactorily explained in terms of known zinc proteins listed in Table II. Karlers and Woodbury[108] demonstrated the presence of carbonic anhydrase (molecular weight ~ 30,000) in the soluble fraction of brain tissue. From their data and additional information from Maren[109] one can calculate that this zinc enzyme contributes substantially to this zinc peak in the elution curve. Equally important is the contribution from the zinc contained in cytocuprein. The presence of equimolar concentrations of copper and zinc in this protein has been demonstrated recently by Carrico and Deutsch[66]. A direct comparison of the copper and zinc content in two peaks in the molecular weight of 34,000 region in Fig. IX shows that 42% of the zinc come from cytocuprein. Again, about 70 μg of cytocuprein contributed to the zinc peak. Both carbonic anhydrase and cytocuprein are stable in the presence of 0.01 M EDTA. Carboxypeptidase has also been reported to be present in brain tissue[76]. However, it should not contribute to this peak because of its low solubility in dilute salt solutions[110].

Carbonic anhydrase was the first zinc metallo-enzyme to be discovered[111]. It contains one gram atom of zinc per mole of protein with a molecular weight of 30,000. Though indirect methods have shown that cysteinyl residues may take part in the metal binding site[112, 113, 114], the actual donor atoms of the zinc-binding site have not been reported as yet.

The first zinc peak accounts for 5% of total zinc content of ~ 87,000. This peak can also be satisfactorily explained in terms of known zinc proteins listed in Table III. Raskin and Sokoloff[68] reported that a significant level of alcohol dehydrogenase activity has been demonstrated in the soluble fraction of rat brain. This zinc enzyme which contains 4 gram atoms of zinc per mole of protein (molecular weight ~ 87,000) catalyzes the oxidation of ethanol or the reduction of acetaldehyde using NAD as a co-factor. This peak is extremely close to the exclusion limit. Another zinc enzyme - lactic dehydrogenase (molecular weight ~ 137,000) may also contribute to it based on the work of Johnson[115] and McGeer et al.[116]. Both alcohol dehydrogenase and lactic dehydrogenase are present in the soluble fraction of brain tissue.

In order to show that the zinc in basal ganglia was indeed protein bound the system was studied by the combination of enzyme hydrolysis, gel chromatography and neutron activation analysis. The results are shown in Fig. XII. Fig. XIIa shows the result in the absence of enzyme hydrolysis. Fig. XIIb shows that only 8% of the zinc left in the protein region after pronase hydrolysis for 44 hours. A separate experiment has indicated that only 7% of the zinc were left in the protein region after

papain hydrolysis for 36 hours.

The choice of basal ganglia for the experiments described above is, of course, somewhat arbitrary. It was based on the comparatively high metal content and also its easily availability. Because of the high concentrations of trace metals in the pineal body, the same techniques have been applied to study its soluble metallo-proteins of copper, manganese and zinc in the next section.

4.7. Identification of Soluble Metallo-Proteins in the Pineal Body

The results of the analysis for soluble metallo-proteins in the pineal body are shown in Fig. XIV. This graph which is similar to Fig. IX shows the presence of copper proteins of apparent molecular weights of $> 90,000$, $\sim 31,000$, $\sim 9,000$ and $\sim 7,000$; a manganese protein of molecular weight of $\sim 62,000$ and zinc proteins with molecular weights of $\sim 87,000$ and $\sim 28,000$. These metallo-proteins appear to be identical to those found in the basal ganglia (Fig. IX). Table VI shows that the pineal body contains the largest total content of the three trace elements. Again, Table XI shows that this high concentration is mainly due to an increase of the metal amounts in the soluble fraction. The same kinds of metallo-proteins were found in both the basal ganglia and the pineal body except that Fig. XIV (the pineal body) shows the metal peaks stronger. It is obvious that these higher peaks are the result of the high concentrations of the three trace metals in the soluble fraction.

4.7.1. Soluble Copper Proteins

It is evident from Fig. XIV that the majority of the copper (~ 72%) in the soluble fraction of the pineal body occurs as metallo-proteins with molecular weights of ~ 9,000 and ~ 7,000 respectively. These metallo-proteins are also formed in the basal ganglia *in vivo* (Fig. IX) and *in vitro* (Fig. IV). In these graphs there are indications that shoulders exist on the low molecular weight side and the peaks are not "Gaussian-shaped". In the basal ganglia, only 57% of total copper is found in the peak with molecular weight of 10,000. The additional 15% of the total copper in the pineal body may contribute to the shoulder in Fig. XIV. This peak (Fig. XIV) can be resolved to more than one peak indicating that at least part of the copper in the pineal body is bound to a protein with a molecular weight of ~ 7,000.

The second largest peak accounts for 16% of the total copper in an elution position corresponding to a molecular weight of ~ 31,000. Based on the apparent molecular weight, this copper protein is the so-called cytocuprein. In this work, 18% and 16% of total copper content contribute to the copper peak corresponding to cytocuprein in the basal ganglia and the pineal body respectively. This indicates that the relative amounts of cytocuprein in the basal ganglia and the pineal body are the same (Table XII). On the basis of the given data, one can calculate that about 140 μg of cytocuprein contributes to the copper peak in Fig. XIV.

The third peak accounts for ~ 5% of total copper in an elution position corresponding to a molecular weight of > 90,000. It is difficult to inter-

TABLE XII

Comparison Between the Cytocuprein in the Soluble Fractions of the Pineal Body and Basal Ganglia

	Ratio Pineal Body	to	Basal Ganglia
Percent of extractable copper (of Table XI)	~ 2	:	1
Weight of tissue homogenized	~ 1	:	2
Weight of extracts fractionated	~ 2	:	1
Overall	~ 2	:	1
Weight of cytocuprein in Fig. XIV and Fig. IX	~ 2	:	1

pret. Since it appeared extremely close to the void volume no molecular weight can be assigned. In fact, it could represent the sum of two or even more copper proteins of molecular weight greater than 90,000. If one examines Fig. IX and XIV, one will find that this peak contributes 15% of total copper content in the basal ganglia (Fig. IX), whereas only 5% of it is found in the pineal body (Fig. XIV). As indicated on page 83, the most likely explanation at the present time is that most if not all of this peak is due to ceruloplasmin (molecular weight ~151,000, containing 8 gram atoms per mole of protein) from blood serum. Since there are more blood vessels in basal ganglia than in the pineal body it is reasonable to believe that there will be more ceruloplasmin in the basal ganglia than in the pineal body. Furthermore, Friede indicated that ceruloplasmin is also involved in the brain metabolism[107].

4.7.2. Soluble Manganese Protein

The gel chromatography-activation analysis experiment shows the presence of one manganese protein with a molecular weight of ~ 62,000 in the soluble fraction of pineal body (Fig. XIV). This manganese protein which accounts for 15% of total manganese is not specific to the pineal body. The work reported earlier indicates that there is a manganese protein with a molecular weight of ~ 61,000 in the basal ganglia. Based on the apparent molecular weight, these two manganese proteins are identical within the experimental error. The relative amounts of the manganese proteins in these two areas are roughly the same (i.e. 20% and 15% respectively). This indicates that there is no drastic change in the

basal ganglia and the pineal body in this respect.

4.7.3. Soluble Zinc Proteins

The distribution of zinc in the soluble fraction of the pineal body is shown in Fig. XIV. Because of the high percentage of extractable zinc in the pineal body (of Table XI), this graph looks better than Fig. IX. It shows the presence of zinc proteins of molecular weights of ~ 87,000 and ~ 28,000.

Most of the protein bound zinc (~ 9% of total zinc) appears in the elution position corresponding to a molecular weight of ~ 28,000. In reference to the discussion on zinc proteins in basal ganglia extract, it is reasonable to believe that this peak is due to carbonic anhydrase and cytocuprein. A direct comparison of the copper and zinc content in two peaks in the molecular weight of 30,000 region in Fig. XIV shows that 39% of zinc come from cytocuprein. The rest (61%) of the zinc peak is due to carbonic anhydrase. This agrees with the result in the basal ganglia (Fig. IX) in which 42% and 58% of the zinc peak are due to cytocuprein and carbonic anhydrase respectively.

The first zinc peak which contributes to ~ 7% of total zinc appears in the elution position corresponding to a molecular weight of ~ 87,000. It is not specific to the pineal body. On the basis of apparent molecular weight, this zinc protein is identical to the one found in the basal ganglia (Fig. IX). It is likely to be alcohol dehydrogenase based on the work of Raskin and Sokoloff.[68] The relative amounts of these zinc proteins in both basal ganglia and the pineal body are roughly the same.

CONCLUSIONS

1. The distribution of copper, manganese and zinc is inhomogeneous throughout the brain.

2. The concentrations of these three elements in the pineal body are relatively high especially in young mammals.

3. The concentrations appear to be related to age.

4. The relative amounts of copper, manganese and zinc which can be extracted from homogenized tissue of different regions of the brain are essentially constant (~ 40%) except for the pineal body. About 70%-80% of the three trace metals in the pineal body were found in the soluble fraction.

5. Several metallo-proteins of copper, manganese and zinc were found in the soluble fraction of brain tissue. On the basis of apparent molecular weights and published data, they are believed to be cytocuprein, ceruloplasmin, carbonic anhydrase, alcohol dehydrogenase, a manganese protein with apparent molecular weight of ~ 61,000 and at least two copper proteins with apparent molecular weights of ~ 9,000 and ~ 7,000. The latter may be specific to brain tissue. All the soluble metallo-proteins of copper, manganese and zinc form only *in vivo* except the copper proteins with apparent molecular weights of ~ 9,000 and ~ 7,000 which form both *in vivo* and *in vitro*.

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