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A SYSTEMATIC STUDY OF BIOCHEMICAL EFFECTS OF HEAVY METAL POLLUTION

Programme of the Research and Preliminary Results on Cadmium and Lead

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A SYSTEMATIC STUDY OF BIOCHEMICAL EFFECTS OF HEAVY METAL POLLUTION

Programme of the Research and Preliminary Results on Cadmium and Lead

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INDEX

PROGRAMME OF THE RESEARCH 5

1. Introduction 5
2. Choice of Pollutant Metals 6
3. Outline of the Research 11
3.1 Preparation of the Radiotracers 12
3.2 Administration of the Radiotracers 13
3.3 Sacrifice, Dissection and Counting of Isolated Organs 13
3.4 Separation of Subcellular Fractions by Differential Centrifugation 14
3.5 Purification of Subcellular Organelles from Subcellular Fractions 14
3.6 Fractionation of Purified Organelles into their Components 14
3.7 Chromatographic Separations on Organelles Components 15
3.8 Analytical and Preparative Electrophoresis 15
3.9 Characterization of the Metal Binding Component and *in vitro* Studies 15
3.10 *In vivo* Studies 16

STATE OF ADVANCEMENT AND PRELIMINARY RESULTS 17

4. Set-up of Stabularium and Cold Room Radiochemical Facilities 17
5. Preparation of Radiotracers with High Specific Activity 17
6. Preliminary Results on Biochemical Effects of Cadmium 18
6.1 Isolation, Purification, Metal Content and "De Novo" Biosynthesis of Rat Liver ^{109}Cd and ^{35}S-double Labelled Cadmium Binding Protein (Cd-BP). 18
6.2 Identification of Cd-BP in Rat Testicles and Spleen 20
6.3 Incorporation of Radioactive Metals into Cd-BP 20
6.4 The Biosynthesis of Rat Liver Cd-BP 21
6.4.1 Effects of Heavy Metals on the Biosynthesis of Cd-BP 21
6.4.2 The Biosynthesis of Cd-BP in Short-Term Experiments: Normal Level of Cd-BP in the Rat 21
7. Distribution of High Specific Activity Radiotracers in the Subcellular 22

Fractions from Rat Liver: Systematic Study in the Soluble Fraction 22

8. Preliminary Results on Biochemical Effects of Lead 23

8.1 Preparations and Counting of ^{203}Pb 23

8.2 Distribution of ^{203}Pb in Rat Tissues 24

PROGRAMME OF THE RESEARCH

1. INTRODUCTION

The assessment of the biological effects on man by his daily exposure to the heavy metal pollution of the environment would require a comprehensive knowledge of the biochemical action of pollutants at the concentration in which they are normally present in polluted environments.

However, the experimental difficulties associated in long-term experiments carried out with metal concentrations which are usually in the part per billion range, are rather high, and in practice most of the knowledge available on biochemical effects of pollutants is based upon short-term experiments, carried out with concentrations of heavy metals which are much higher than those present in polluted environments.

Differences in systematic manifestations between acute and chronic exposure have been experimentally found, but the basic mechanisms involved are still far from being explained, even for elements of major concern such as Cd, Pb, Hg.[1, 2].

Among the few studies reported on the binding of metals at environmental levels with specific cellular components and their biotransformations are those reported on metallothionein (a specific protein of liver and kidney which might act as a detoxication agent for cadmium) and nickeloplasmin, (a serum protein which was found to bind large amounts of nickel)[3, 4, 5].

For a large number of heavy metals which might be classified as pollutants of minor concern, only a few data are available on their biochemical effects, even at more massive doses.

It may therefore be stated that:

- there is a need to distinguish, at least for pollutants of major concern such as Cd, Cr, Hg, Pb and Zn, between the biochemical effects of acute exposures and those of chronic exposures as may result from daily expo-

sures to a polluted environment;

- there is a need to provide more systematic information on the biochemical effects of pollutants which were reported as possible environmental contaminants and for which a few biochemical effects were demonstrated.

Such studies are greatly hindered by experimental difficulties such as:

- the necessity of "labelling" for in vivo studies nanogram or sub-nanogram amounts of metals (a problem which may be difficult even for very sensitive techniques such as radioactive tracing);
- the necessity of detecting and measuring trace amounts of metals in microsamples of cellular components which are obtained after long and complex separation procedures.

2. CHOICE OF POLLUTANT METALS

We have selected 5 elements (Cd, Zn, Se, Hg and Cr) for the long term experiments and 4 elements (Pb, V, Ni, Be) for the short term experiments. We also planned to carry out some works on As, Tl, Mo, Te, Bi and Sn, which are presently receiving much attention[6, 7, 8, 9, 10, 11] for their impact at biochemical level and antagonism or competition with pollutants of major concern.

The choice was not simply based on the concept of biological essentiality and toxicity. In fact, metals differ from most other pollutants in that often they can have an essential biological function in addition to a toxic role. As an example Zn and Se, which are essential as constituent of alkaline phosphatase[12] and glutatione peroxidase[13] enzymes when present in excessive quantities, are as toxic as Cd, Hg and Pb which are known as environmental contaminants without essential biological function.

General criteria to determine which elements are most likely to pollute

the environment, have been recently reported. Wood[14] classified pollutants elements as:

1) non critical (Fe, Si, Rb, Al, Na, K, Mg, Ca, P, S, Cl, Br, F, Li, Sr);

2) very toxic and relatively accessible (Be, Co, Ni, Cu, Zn, Sn, As, Se, Te, Pd, Ag, Cd, Pt, Au, Hg, Tl, Pb, Sb, Bi);

3) toxic but very insoluble (Ti, Hf, Zr, Re, W, Nb, Ta, Ga, La, Ir, Os, Ru, Ba).

Fishbein[15] reported a list of pollutants which may pose health hazard in the environment (Cd, Se, Hg, Pb, Be, V, As, Mn, B, Y, Sb, Sn, Ge, Zr, Bi).

The essential present knowledge on the behaviour at cellular level of the heavy metals which were chosen in the present research are summarized below.

Cadmium

Cd progressively accumulated in the human organism with age, particularly in the kidney tissue[16,17]. This is attributed to a protein, metallothionein, (MW = 10,000), with an exceptionally high content of cysteine, which is synthesized following the administration of cadmium[18,19]. After administration via inhalation, ingestion or injection, cadmium is transported bound to both plasma and erythrocites proteins via blood to other tissues in the body. The metal enters the tissues of various body organs, particularly in liver and kidney, although testicles, pancreas and spleen contain it[21]. The biological half-life of cadmium is extremely long and the turnover of metallothionein is practically unknown. Although Piscator suggested that the protein could be continuously synthesized in chronic exposure to cadmium acting as a detoxifying agent for this toxic metal, the metabolic role of metallothionein is far from being understood[22].

Selenium

The highest concentration of selenium in man was found in the liver, followed by the kidney, spleen and lung.
Selenium has been shown to cross the placenta in rats[23]. It could exert its toxic action by the oxidation of sulfhydril metabolites, thus inactivating them. It has also been suggested that selenium can compete with sulphur at those sites in which sulphur normally plays a role in cellular metabolism, although the biosynthesis of selenoaminoacids from selenites in animals is not yet demonstrated[24]. Following absorption selenium is carried fixed in the red blood cells associated with plasma albumin and globulin which transport the metal to more stable binding sites in blood and tissues[25]. Different effects were observed in rats intoxicated with selenates or selenites. It was found that selenate can be reduced to more toxic selenite and that the body could detoxify this latter by converting it in the volatile methyl selenide[26].

Mercury

The highest level of mercury in animals was found in kidneys, followed by liver, spleen and brain. The subcellular distribution of inorganic mercury shows that the metal is accumulated by the lysosomal fraction of liver. However, differences were observed in the subcellular distribution between organic and inorganic mercury compounds[27]. Mercury is found bound to hemoglobin and albumin. The reaction represents an equilibrium involving all the -SH groups of the proteins[28]. The metal is also able to bind to both nuclear proteins and to DNA causing complex structural changes within DNA[29, 30].

Chromium

The subcellular distribution of chromium showed that the soluble fraction of heart, pancreas and adipose tissue have the bulk of chromium in contrast to liver where it mainly accumulates in the microsomes.
The distribution in liver fractions was altered by induced diabetes or by the

feeding of high fat diets. In particular, when the rate of hepatic lipogenesis was normal or elevated, chromium appeared to move from the nuclear to the microsomial fraction of liver[31].

Cr(VI) has been recognized as the toxic form while Cr(III) can form a complex with sulfhydril groups on the A-chain insulin acting as a cofactor of insulin action[32, 33]. It was shown that chromium was associated with RNA in nucleic acid metabolism[34].

Lead

Lead metabolism follows closely that of calcium, particularly in deposition and mobilization from bone. After an acute exposure, lead is found in the liver and kidney[35].

At the cellular level the best known effect of lead is the inhibition of the enzymes which depend on -SH groups for their function. The metal is implicated in the metabolism of δ-aminolevulinic acid (ALA) and therefore in the biosynthesis of heme from iron and protoporphyrin[36].
Different results were reported in the literature on the subcellular distribution of lead[37, 38]. Baltrop shows that the metal is contained in the soluble and mitochondrial fractions, while it was bound little to the lysosomes[38].

Blood lead is found mainly associated with the erytrocytes. However, the binding site on the hemoglobin molecule is not known also if it was suggested that free -SH groups are not essential in the formation of the lead-hemoglobin complex[39].

Beryllium

Beryllium accumulates in all cells producing an insoluble beryllium phosphate removed from the circulating blood by the reticuloendothelial system of the liver[40].

Beryllium concentrates at subcellular level into lysosomes and nuclei of cells. In particular it binds to rat liver nuclei with marked affinity and inhi-

bition of the enzymes required for DNA synthesis but with no effect on RNA metabolism. However, the nature of the binding site for beryllium in the nucleus is unknown[41]. Inhibition studies have shown that alkaline phosphatase, phosphoglucomutase and adenosine phosphatase have high affinity for beryllium which reacts with the enzymes in a very specific way[42]. It was shown that under certain circumstances beryllium is a carcinogen[43].

Vanadium

Different effects were observed in animals intoxicated with vanadium compounds, the most toxic form being the pentavalent followed by tri- and divalent.

The established effects of vanadium salts at molecular level are:

- reduction of oxygen transport combining it with hemoglobin[44];
- inhibition of cholesterol biosynthesis at concentrations of 5×10^{-5} M and lowering of phospholipids in the blood[45];
- oxidation of serotonin in vitro[46];
- inhibition of calf intestine alkaline phosphatase[47].

Recently, it has been shown that the toxic effect of vanadium in the rat is unrelated to Mo-antagonism and it is demonstrated that these effects are related to accumulation of the metal in the liver and in the kidneys[48].

Nickel

The subcellular distribution of dietary nickel shows that it accumulates in the soluble fraction of the liver and in soluble fraction and nuclei of kidney[49]. Injections of $^{63}NiCl_2$ in rabbits show that serum ^{63}Ni can be separated into three components: α-macroglobulin-bound ^{63}Ni (nickeloplasmin), albumin-bound ^{63}Ni and ultrafiltrable ^{63}Ni. The subcellular distribution of $^{63}Ni(CO)_4$ shows that ^{63}Ni in the subcellular fractions of liver and lung was approximately proportional to the dry weights of the respec-

tive fractions, the metal being bound to RNA, DNA and proteins[50].
A significant increase in the incidence of malignant tumors was observed in rats receiving i.v. injections of nickel carbonyl. An inhibition of enzymes induction in lung and liver was observed[51].

3. OUTLINE OF THE RESEARCH

The research outlined below aims at the study, under systematic operation conditions, of the distribution of labelled metal pollutants at low-dose in organs, subcellular fractions and isolated and fractioned components of subcellular fractions in view of identifying specific metal binding component.

The complete research for each metal involves the following steps.

3.1 Preparation of radiotracers

3.2 Administration of the tracer to a group of animals

3.3 Sacrifice of the animals at various intervals of time, dissection and radioactivity evaluation in different organs

3.4 Separation of subcellular fractions by differential centrifugation

3.5 Purification of organelles from subcellular fractions

3.6 Fractionation of purified organelles into their components

3.7 Chromatographic separation on organelles components

3.8 Isolation of individual metal binding components by analytical and preparative electrophoresis on polyacrilamide gels.

3.9 Characterization of the metal binding component and *in vitro* studies

3.10 *In vivo* studies

Two types of experiments are envisaged, here called:

Long-term experiments: (up to 3-4 months) the labelled metal will be admi-

nistered via drinking water at low concentrations (environmental levels) over long periods of time in order to simulate as close as possible the conditions of polluted environments.
Long-term experiments will be carried out on groups of 100 rats, sacrificed in groups of 10 animals at various intervals of time. A detailed scheme of the various experimental works which will be carried out for each metal is given in Table 1. The procedure described is the most complete one. Adaptations will be done as long as the research proceeds to eliminate the non-essential steps.

Short-term experiments: (1-7 days) the labelled metal will be injected at acute or subacute levels.
They will be carried out on a small number of animals as preliminary orientating experiments or to evidentiate differences between chronic and acute effects.

3.1 Preparation of the Radiotracers

The systematic study is made possible by the availability of radiotracers, radiochemical facilities and highly selective measuring techniques, not usually available to biochemical laboratories and which make it possible to label in vivo and in vitro very minute amounts of pollutant metal. The radiotracers of each element were selected on the basis of:

1) possibility of obtaining very high specific activity,
2) half-life sufficiently long for long-term experiments,
3) easy detection of the emitted radiation.

The preparations are essentially done:

a) by proton irradiations in a cyclotron,
b) by neutron irradiations in a nuclear reactor.
Radioisotopes production in a nuclear reactor by (n,γ) reaction and optimum conditions for producing several isotopes have been already deve-

loped during previous years. An irradiation facility will become available during 1975 by the installation of a hydraulic irradiation channel in the ESSOR reactor with a neutron thermal flux of 3.5×10^{14} neutrons cm^{-2} sec^{-1}. By its use radioisotopes will be prepared both with very high specific activity and using nuclear reactions other than (n, γ). Table 2 shows the nuclear data of the radioisotopes proposed for this study.

3.2 Administration of the Radiotracers

The concentrated solutions of the radiotracers obtained in the preceeding step will be stored in a refrigerator. Dilution for long-term experiments will be carried out with well-characterized mineral water. Exhaustive preliminary experiments will be done to make sure that the tracer is taken by the animal in the desidered chemical form and that uncertainty of the chemical state, lack of equilibrium of the diluted solution and absorption effects do not induce misleading experimental artifacts.

3.3 Sacrifice, Dissection and Counting of Isolated Organs

The animals will be sacrificed by heart puncture after ether anaesthesia. The following tissues will be separated: brain, heart, kidney, liver, spleen, muscle, lung, thyroid, testes, bone, blood, stomach, G. I. tract.

The organs will be weighed, washed with buffer solution (a buffer perfusion will be done for liver or other organs, if found necessary) and homogenized. Homogenization will be done in a refrigerated and shielded homogenizer which ensures maximum safety in case of rupture of the organ container.

Sacrifice and dissection will be carried out as rapidly as possible. The organs will be kept refrigerated by ice baths, in order to minimize biochemical reactions inducing migration of metal ions among various subcellular fractions before homogenization.

Aliquots of the homogenized organs will be taken for radioactivity measurements while the rest of it will be frozen and stored for further handling.

3.4 Separation of Subcellular Fractions by Differential Centrifugation

The separation of subcellular fractions will be carried out on those organs which showed the most significant accumulation of the radiotracers. Liver and kidney will always be considered. Cellular fractionation will be carried out in two steps:

1) Nuclei and mitochondria by preparative centrifuge,
2) Lysosomes, microsomes and soluble cytoplasmatic fraction by preparative ultracentrifuge.

The procedures adopted will be the classical ones, reported in the literature. Fig. 1 shows the one for rat liver.

Aliquots from the fractions obtained will be taken for radioactivity evaluation, the rest will be frozen and stored for further handling.

3.5 Purification of Subcellular Organelles from Subcellular Fractions

Purification of the subcellular organelles will be carried out in order to minimize the effects of misleading cross-contaminations. The most recent procedures reported in the literature will be applied. The flow-sheets of a few of them (nuclei, mitochondriae, microsomes) are shown in Fig. 1.

3.6 Fractionation of Purified Organelles into their Components

The organelles which will show a significant accumulation of the tracer will be submitted to procedures for the isolation of their components. The most recent procedures reported in the literature will be applied. The flow-sheets of a few of them (isolation and separation of subnuclear components, fractionation of mitochondria and Golgi apparatus are shown in Fig. 2. They will mostly be carried out in a cold room area at $+4^{\circ}C$.

3.7 Chromatographic Separations on Organelles Components

The most significant organelles components will be submitted to a successive fractionation into components of different molecular weight by gel chromatography. In-line detection systems allow a continuous measurement of radioactivity and U. V. absorption at two different wavelengths for continuous analysis of metal and protein content. The effluent will be collected into separate fractions on which tests for specific enzymatic activities will eventually be carried out.

The most significant fractions will be pooled desalted by means of hallow fibres, lyophilized, and kept for further handling.
The presence of metals other than the ones labelled will be detected and quantitatively estimated by activation analysis of microsamples of the lyophilized product.

3.8 Analytical and Preparative Electrophoresis

Analytical gel electrophoresis will be carried out on the most significant protein fractions. U. V. scanning of the gel will allow to isolate "electrophoretically pure" proteins. The gel will then be divided into 1-2 mm slices and the radioactivity profile will be measured. Activation analysis of individual gel slices will allow identification and, possibly, quantitative estimate of metals other than the one labelled.

Preparative electrophoresis will be carried out on the most significant fractions in order to obtain larger quantities of specific metal-binding components for full characterization and successive in vitro studies.

3.9 Characterization of the Metal-Binding Component and in Vitro Studies

Studies in vitro to characterize the metal-binding component identified by the systematic study outlined above will also be carried out:

- elemental composition,

- stoichiometric ratios,
- identification of metal-binding sites,
- saturation levels,
- synergistic and antagonistic effects of other pollutants.

Greatest advantage will be taken of an automatic dialyser being under construction in order to minimize the operators time and to diminish the risk arising to persons from relatively high radiation levels.

In particular the dialyser will allow the study in vitro of the interaction of heavy metals with metalloenzymes and nucleic acids, already well known as potential metal binding sites. It is known that heavy metals such as Hg and Cd alterate the structure of nucleic acid by interacting with phosphate groups and/or bases[52].
In addition pollutant metals could interfere with the catalytic function by displacing the essential native metal of metalloenzymes[53].

Because the extent of binding of metals to nucleic acids is extremely low and since the research on metalloenzymes must necessarily be carried out on very minute amounts of materials the availability of metals in radioactive form with high specific activity is of very great help in these studies.

3.10 In Vivo Studies

The following particular studies will be carried out in vivo after identification of the metal-binding component:

- parameters which affect the accumulation of metals in the body,
- influence of the chemical forms of the metal,
- influence of the metal in the biosynthesis of the metal-binding component,
- synergistic and antagonistic effects of other pollutants.

STATE OF ADVANCEMENT AND PRELIMINARY RESULTS

4. SET UP OF STABULARIUM AND COLD ROOM RADIOCHEMICAL FACILITIES

Special modular type cages to minimize metal contamination have been developed. A particular water-supply device was developed in order to minimize radioactive contamination of cages and animals. The drops of water lost during the drinking process are recovered into a polyethylene bottle.
Preliminary experiments showed that after administration of ^{109}Cd to rats the radioactive contamination in all the parts of the cage are insignificant. Feces and urines are collected into a polyethylene container which can be directly counted to obtain data on the excretion of radiotracers.

A cold room (area at + 4°C) radiochemical facility has been set up in order to minimize temperature depending artifacts during the fractionation of the purified cellular organelles into their components. The cold room has been equipped with gel chromatography (flow analyzers for monitoring UV absorption at 280 and 254 nm, fully automatic fraction collectors, recorders, peristaltic pumps and columnsof different size). The cold area has been designed and especially equipped with high level of radioactivity as required for some in vitro studies.
Figure 3 shows the stabularium, the various parts of the modular cage and the cold room radiochemical facility.

5. PREPARATION OF RADIOTRACERS WITH HIGH SPECIFIC ACTIVITY

Table 2 gives the nuclear pertinent data of the radioactive isotopes used for the proposed study. Most of them have been prepared carrier-free, by proton irradiation at the cyclotron of the Milan University.
The same laboratory has measured the excitation function for each isotope to provide useful information on the possible contaminants from interfering reactions and to allow a proper choice of the bombarding energy.

The knowledge of the excitation function is also essential to determine whether enriched targets must be used and the degree of enrichment which is necessary. The separation from interfering radionuclides and target element, the preparation for their biological use and the analytical controls have been set up by our laboratory. Figure 4 shows as an example the experimental excitation function for the production of ^{48}V from metallic titanium targets.
A complete description of the production methodology will be reported elsewhere.

6. PRELIMINARY RESULTS ON BIOCHEMICAL EFFECTS OF CADMIUM

Studies on the subcellular distribution of cadmium show that after a single injection the cellular cadmium was almost entirely present in the soluble fraction of liver and kidney[18] bound to a cadmium-binding protein (Cd-BP), a low molecular weight protein with a high number of cysteine residues (21 g atoms/mole of protein[54]). For this the incorporation of ^{35}S-cysteine in the Cd-BP was used to study the biosynthesis of the protein in Cd-treated rats in respect to normal animals. In addition, we have investigated in vivo the interaction of heavy metals with Cd-BP:

- by neutron activation analysis of Cd-BP purified by gel chromatography,
- by incorporation of radiotracers in the protein after administration of labelled metals to Cd-treated rats.

6.1 Isolation, Purification, Metal Content and "De-Novo" Biosynthesis of Rat Liver ^{109}Cd and ^{35}S-double Labelled Cadmium-Binding Protein (Cd-BP)

One group of rats was treated i.p. with ^{109}Cd and ^{35}S-cysteine. The animals were sacrificed by cervical dislocation after 24 h. Liver tissues were homogenized in 0.25 M sucrose. The soluble cytoplasmatic fraction was obtained by ultracentrifugation at 105.000 x g for 90 min. Gel filtra-

tion of soluble fraction was done with 10 mM Tris-HCl buffer, pH=8.2 as eluant on Sephadex G-75 column. UV absorbance of eluate was monitored at 254 and 280 nm by an LKB Uvicord III instrument.
The ^{109}Cd radioactivity in the eluate was measured by an autogamma (NaI(Tl) crystal) while the ^{35}S radioactivity was detected after separation by ion exchange chromatography of ^{109}Cd radioactivity.
All operations were done in the cold room at $4^{o}C$.
Homogeneity of the Cd-BP was verified by disk electrophoresis, while neutron activation analysis was used to determine simultaneously various heavy metals in microsamples of determined protein. The nuclear technique was also used to the metal content in the Cd-BP from gel chromatography after further purification by disk electrophoresis. Electrophoretic separation was performed in duplicate. The first gel was stained for proteins, while the second gel was sliced in small discs, 1 mm thickness, which were examined for their metal content by neutron activation analysis.

The results are shown in Figure 5:

- a new protein peak (Cd-BP) containing all ^{109}Cd radioactivity appears in UV profiles at 254 nm of Cd-treated rats, absent in untreated controls. No protein peak appears at 280 nm (dotted line in the UV profile) because Cd-BP does not contain aromatic aminoacids[54] (Figure 5A);
- a much higher incorporation of ^{35}S-cysteine was found in this peak in respect of controls (Figure 5A);
- the presence of the following elements has been demonstrated by neutron activation analysis in the Cd-BP isolated by gel chromatography: Cd, Zn, Cu, Hg, Ag and traces of Fe, As, Mn, Sb, Sc, Cs and Au. A typical gamma-ray spectrum of the neutron activated rat liver Cd-BP is shown in Figure 5B;
- Cd, Zn, Hg and Cu of the Cd-BP have similar profiles in the gel after disk electrophoresis (Figure 5C). The Cd-BP, which is homogeneous

in terms of molecular weight, seems not to be electrophoretically homogeneous. This was confirmed by measuring also ^{35}S-cysteine radioactivity in the gel: the profile after electrophoresis was similar to that of ^{109}Cd showing a maximum distribution in two peaks.

6.2 Identification of Cd-BP in Rat Testicles and Spleen

To establish if Cd-BP could be present in rat testicles and spleen, one group of 10 rats was treated with ^{109}Cd carrier-free. The animals were sacrificed after 24 h and the soluble fraction isolated and fractioned as described under section 6.1. The results show that all ^{109}Cd radioactivity was present in fractions corresponding to Cd-BP although the protein was too low to be detected in the UV profile.
However, while in liver (Figure 5A) and testicles (Figure 6A) the ^{109}Cd is associated only with Cd-BP of low molecular weight, in the spleen the ^{109}Cd is also present in high molecular weight components (Figure 6B).

6.3 Incorporation of Radioactive Metals into Cd-BP

Recently Nordberg observed that Cd-BP could play a more general role in the metabolism of various heavy metals[1]. For this, to provide systematic information on the relative incorporation into Cd-BP of rat liver under *in vivo* conditions, one group of rats received a single intraperitoneal injection of $^{109}Cd\ Cl_2$ and radioactive metal, while a second group,as a check, received only the radioactive metal like the first group but without $^{109}CdCl_2$. After 24 h the Cd-BP was isolated as described in section 6.1.

From 21 metals tested, only ^{65}Zn, ^{197}Hg, ^{64}Cu, ^{110m}Ag and ^{113}Sn were found incorporated into "de novo" biosynthesized Cd-BP.
Figure 7 shows the results obtained.

6.4 The Biosynthesis of Rat Liver Cd-BP

6.4.1 Effects of Heavy Metals on the Biosynthesis of Cd-BP

In order to prove that the incorporation of cadmium is not affected by any other metal, one group of rats received a single injection of ^{109}Cd and ^{35}S-labelled cysteine in the presence of other metals, while a second group, as a check, received only ^{109}Cd and ^{35}S-labelled cysteine without other metals.

After 24 h the Cd-BP was isolated as described in section 6.1 and the ^{109}Cd and ^{35}S radioactivities measured in the Cd-BP fraction. The results show that both incorporation of cadmium and the biosynthesis of Cd-BP are not influenced by the presence of other metals such as: Be^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Al^{3+}, Au^{3+}, Cr^{3+}, Ga^{3+}, La^{3+}, Ir^{4+}, Sn^{4+}, Zr^{4+}, Ag^{+}, Bi^{3+}, Pb^{2+}, Tl^{+}, U^{6+}, As^{5+}, Mo^{6+}, V^{5+}, Sb^{5+}, Se^{4+}.

6.4.2 The Biosynthesis of Cd-BP in Short-term Experiments: Normal Level of Cd-BP in the Rat

The simultaneous incorporation of ^{109}Cd and ^{35}S-cysteine into Cd-BP and its isolation as described under section 6.1, was used to study the biosynthesis of Cd-BP in subacute exposures to cadmium. Some conclusions seem valid:

- the biosynthesis of Cd-BP is controlled by the cadmium concentration also in the presence of other heavy metals (section 6.4.1);
- when rats are intoxicated daily with amounts of cadmium of 0.8 mg/kg for 8 days, the biosynthesis is linear with the cadmium concentration (Figure 8);
- the daily dietary intake for rats is of the order of 5 µg cadmium (confirmed by neutron activation analysis of the diet). This quantity should be

sufficient to synthesize the amount of Cd-BP corresponding to the fraction adsorbed (6% from literature data[55]). The Cd-BP should therefore be normally present in the rat liver. The hypothesis seems confirmed by the experiments described under section 6.3 and 7 on the incorporation of various metals in the Cd-BP. It was found that a small amount of the different metal ions incorporated in the Cd-BP of Cd-treated rats (^{65}Zn, ^{64}Cu, ^{197}Hg, ^{113}Sn and ^{110m}Ag) is also present in normal animals (without Cd-treatment), probably due to minute amounts of Cd-BP not detectable by UV-measurement.

7. DISTRIBUTION OF HIGH SPECIFIC ACTIVITY RADIOTRACERS IN THE SUBCELLULAR FRACTIONS FROM RAT LIVER: SYSTEMATIC STUDY IN THE SOLUBLE FRACTION

A systematic study of the distribution in rats of radiotracers, shows that liver and kidney are important deposition organs of many heavy metals[56]. The knowledge of the subcellular distribution of heavy metals, which is of great importance to understand the biochemical effects in rats intoxicated with pollutant elements, is far from being investigated. We have systematically examined in vivo the distribution of radiotracers in the soluble fractions of rat liver. Animals were injected i.p. with radioactive metal ions and sacrificed after 24 hours. The soluble fraction of liver homogenate, obtained by centrifugation at 105.000 x g for 90 min, was chromatographed on Sephadex G-75 column (100 x 5 cm) and proteins and radioactivities measured in the collected fractions as described in section 6.1. The results are illustrated in Figure 9:

- all radiotracers were always recovered from the supernatant after Sephadex G-75 chromatography in association with the fraction of high molecular weight components, although ^{201}Tl, ^{51}Cr, ^{7}Be, ^{203}Pb, ^{65}Zn, ^{197}Hg, ^{113}Sn are also present in the fractions corresponding to low molecular weight;
- ^{109}Cd and ^{110m}Ag were present only in the Cd-BP region (MW ≈ 10.000);

- ^{198}Au was also associated with a fraction corresponding to a molecular weight of 5.000 - 6.000;
- ^{64}Cu is associated with a fraction corresponding to Cd-BP and with a more specific protein, probably cytocuprein[57] of molecular weight 30.000 - 35.000.

In conclusion, it appears that high molecular weight components of the soluble fraction of rat liver, are greatly involved in the metabolism of many heavy metals. The study of the biochemical nature of these components will give us informations in view to identify the intracellular metal-binding site(s).

8. PRELIMINARY RESULTS ON BIOCHEMICAL EFFECTS OF LEAD

Di Ferrante and Bordeau [58] recently reviewed the distribution of stable and radioactive lead (^{210}Pb) in different organs of man and concluded that data on the distribution of lead in the different compartments of the organism are far from being consistent. In addition, very few works concern the subcellular distribution of lead[37, 38]. The purpose of this study was to investigate the distribution of ^{203}Pb in organs, subcellular fractions, components of purified subcellular fractions and molecular components of fractioned subcellular fractions. The study has been carried out by means of ^{203}Pb radioisotope which has favourable nuclear characteristics for short-term experiments[59]. The use of ^{210}Pb in the study of lead metabolism is limited by the high toxicity of the daughter ^{210}Po and by the difficulty of measuring accurately the counting rate of the emitted α-radiation.

8.1 Preparation and Counting of ^{203}Pb

Fig. 10 shows the excitation function for the production of ^{203}Pb by proton irradiation of thallium target . The purification of ^{203}Pb was carried out by double coprecipitation with ferric hydroxide and subsequent extraction of iron by propylether. Further purification from thallium traces

was performed by cation exchange resin.
All measurements of ^{203}Pb radioactivity were done by γ-ray spectroscopy with a Ge(Li) detector at the characteristic line of 279 KeV.

8.2 Distribution of ^{203}Pb in Rat Tissues

18 μg of stable lead nitrate/rat plus 700 μCi of ^{203}Pb carrier-free/rat were injected i.v. to eight animals. The rats were sacrificed after 24 h. by heart puncture after ether anaesthesia. Blood was collected and the organs removed and homogenized. Aliquots of homogenates were directly counted for ^{203}Pb radioactivity. The results are reported in Fig. 11.

- Subcellular distribution of ^{203}Pb in rat liver and kidney: gel chromatography of soluble fractions from liver, kidney and spleen

The homogenates of liver and kidney were fractioned by differential centrifugation. The ^{203}Pb radioactivity was measured in nuclear, mitochondrial, lysosomal, microsomal and soluble fractions. Further purification of nuclei and mitochondria has been carried out (see procedures in Fig. 1). The results are shown in Fig. 11 and 12. The soluble fractions from kidney, liver and spleen were chromatographed on Sephadex G-75 column as reported for the isolation of Cd-BP (see section 6.1). The UV adsorbance and ^{203}Pb radioactivity profiles from gel chromatography are shown in Fig. 13.

- Distribution of ^{203}Pb in the components of nuclei and mitochondria from liver and kidney

Purified liver and kidney nuclei and mitochondria were respectively fractioned in membrane and "bulk chromatine" (nuclei) and soluble, interface, light and heavy fractions (mitochondriae) (see procedures in Fig. 2). The ^{203}Pb radioactivity was measured in these fractions (Fig. 11 and 12).

- Chromatographic separation of liver and kidney nuclear chromatine and soluble mitochondrial fractions

The nuclear "bulk chromatine" and the mitochondrial soluble fraction from liver and kidney were chromatographed on Sephadex G-75 column and the UV absorbance and ^{203}Pb radioactivity profiles were measured. The results are shown in Fig. 11 and 12.

The following conclusions can be drawn from the experiments which were described above:

- Rat kidney and liver are the organs in which most of ^{203}Pb can be recovered after 24 h. from a single i.v. injection.
- The subcellular distribution of ^{203}Pb shows that most of the radiolead was contained in the nuclear and soluble fractions of kidney and liver. Mitochondria of kidneys bind much more lead than those of liver.
- Gel chromatography of soluble fractions from liver, kidney and spleen show that ^{203}Pb are associated almost with fractions corresponding to high molecular components.
- About 65-70% of ^{203}Pb in nuclei is associated with membrane, while about 30% is present in the chromatine. In this latter case radiolead is present in one protein peak.
- Different distribution of ^{203}Pb in the mitochondria components of kidney and liver were obtained. However, gel chromatography of mitochondrial soluble fractions shows association of ^{203}Pb radioactivity with fractions corresponding to high molecular weights.

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TABLE 1		FLOW-SHEET OF SYSTEMATIC STUDY
Day	Number of Remaining Animals	
	0	Preliminary experiments - preparation of tracer - valence state tests - stability of conc. and diluted solutions
0	100	Systematic Run start, ten animals are sacrificed at interval of ten days
10	90	
20	80	
30	70	
40	60	obtaining data on cumulation of tracer in organs, cellular fractions, isolated organelles and their components. On cumulated fractions make gel chromatography separations
50	50	
60	40	
70	30	
80	20	
90	10	
100	-	
		Final characterization and in vitro studies - analytical electrophoresis - characterization (elemental composition, stoichiometric ratios, binding sites...) - in vitro studies

TABLE 2 RADIOISOTOPES INVOLVED IN THE SYSTEMATIC STUDY

Pollutant and type of experiment	Isotope	T 1/2	Source	Principal emission (MeV)	Radiochemical separation and Specific Activity
Long-term experiments	Cd ^{115m}Cd	43 d.	Nucl. react.	0.49 0.94	1 mCi/mg Cd
	Cd ^{109}Cd	450 d.	Cyclotron	0.022 Ag X-ray 0.088 via 40 sec ^{109m}Ag	Carrier-free. Solvent extraction of radio-cadmium and anion exchange chromatography
	Zn ^{65}Zn	245 d.	"	0.51 from β^+ 1.15	Carrier-free Anion exchange chromatography
	Cr ^{51}Cr	27.8 d.	"	0.32	Carrier-free. Coprecipitation of radio-chromium and ion exchange chromatography
	Hg ^{203}Hg	46.5 d.	Nucl. react.	0.28	1 mCi/mg Hg
	Se ^{75}Se	127 d.	" "	0.14 0.27	10 mCi/mg Se
Short-term experiments	Pb ^{203}Pb	2.17 d.	Cyclotron	0.28 0.40	Carrier-free. Coprecipitation of ^{203}Pb by Fe^{3+} and solvent extraction of iron by ether
	Be ^{7}Be	53.6 d.	"	0.48	Anion exchange chromatography
	V ^{48}V	16.2 d.	"	0.99 1.31	
	^{49}V	330 d.		0.005 Ti X-ray	Anion exchange chromatography
	Ni ^{63}Ni	80 y.	Nucl. react.	0.067	5 mCi/mg Ni

CELL FRACTIONATION OF RAT LIVER

Tissue homogenate
(0.25 M Sucrose, Tris-HCl pH=7.2)

↓ Filtration by cheese cloth

↓ 700 × g, 10 min → Residue: Crude NUCLEI

Supernatant

↓ 9000 × g, 10 min → Residue: Crude MITOCHONDRIA

Supernatant

↓ 30.000 × g, 25 min → Residue: Crude LYSOSOMES

Supernatant

↓ 105.000 × g, 100 min → Residue: MICROSOMIAL fraction

Supernatant → Soluble CYTOPLASMATIC fraction

PURIFICATION OF RAT LIVER NUCLEI

(W.W. Franke, Exptl Cell Res. (1973). 81, 365)

Resuspend "Crude NUCLEI" in
0.45 M Sucrose, 0.07 M KCl, 0.01 Tris-HCl pH=7.2

↓ Add 7 volumes of "dense sucrose solution"
(2.0 M Sucrose, 0.07 M KCl, 0.01 Tris-HCl pH=7.2)

↓ Layer over a large volume of "dense sucrose solution"

↓ 22000 × g, 70 min → Supernatant: Discard

Resuspend in 0.3 M Sucrose, 0.07 M KCl, 0.01 Tris HCl pH=7.2

↓ Adjust sucrose concentration to 1.8 M with "dense medium"

↓ Layer over an equal volume of "dense solution"

↓ 60000 × g, 90 min → Supernatant: Discard

Residue: PURIFIED NUCLEI.

PURIFICATION OF RAT LIVER MITOCHONDRIA

(G.L. Sottocasa, J. of Cell Biol. (1967), 32, 415)

Resuspend "Crude MITOCHONDRIA" in
0.25 M Sucrose

↓ 24000 × g, 10 min → Supernatant: Discard

Residue (mitochondria plus other pellets)

↓ Resuspend and layer over a sucrose gradient
(1.8 - 2.2 M Sucrose)

↓ 16000 × g, 25 min (Swinging rotor) → Supernatant: Discard

Residue: PURIFIED MITOCHONDRIA

PURIFICATION OF RAT LIVER MICROSOMES

(J.J.M. Bergeron, J. of Cell Biol. (1973), 59, 73)

Resuspend "MICROSOMIAL fraction" in
0.25 M Sucrose

↓ 105.000 × g, 60 min → Supernatant: Discard

Residue

↓ Resuspend in 0.06 M Tris-HCl pH=8.5

↓ Pass twice through French press

↓ Adjust sucrose concentration to 0.25 M

↓ 105.000 × g, 60 min → Supernatant: MICROSOMIAL CONTENT

Residue: MICROSOMIAL MEMBRANES

Figure 1

FRACTIONATION OF RAT LIVER MITOCHONDRIA

Suspend purified mitochondria in 0.25 M Sucrose

Sonification (G.L. Sottocasa, J. of Cell Biol. (1967), 32, 415)

Layer over a solution of 1.18M Sucrose

24.000 × g, 180 min (Swinging rotor)

- heavy fraction → Mitochondria with a more or less well preserved inner structure but without the outer membrane. Inner membrane system and part of the matrix containing the bulk of respiratory chain enzymes
- light → Vesciculated derivatives of the outer membrane. Enzymes of this fraction are not from the outer membrane
- soluble fraction → Material present between the two mitochondrial membranes. Part of the matrix. Material released from both the inner and outer membranes

FRACTIONATION OF RAT LIVER GOLGI APPARATUS

Supernatant of liver homogenate (10000 × g, 10 min)

105.000 × g, 90 min → Supernatant (Discard)

(J.H. EHRENREICH, J. of Cell Biol. (1973) 59, 45)

Residue: resuspend in 1.15M sucrose

Load under discontinuous gradient

64.000 × g, 200 min

- Pellet. Rought microsomes
- Load smooth microsomes
- 1.15-0.86M GF_3 → Residue GF_3 membrane
- 0.86-0.60 M GF_2 → Residue GF_2 membrane
- 0.66-0.25 M GF_1 → Residue GF_1 membrane

FRACTIONATION OF NUCLEAR COMPONENTS

Purified nuclei (W.W. Franke, Exptl. Cell. Res. (1973), 81, 365)

Suspend in 0.1M sucrose and Tris-HCl, pH = 7.4

Sonification

Add "high salt extraction medium" (0.3M sucrose, 1.15M KCl, 0.01M Tris-HCl, pH=7.2)

Stirrer for 3-4 hours at 4°C

110.000 × g, 120 min → Supernatant (BULK NUCLEAR CHROMATIN)

Residue: resuspend in 0.1M Sucrose, 0.07M KCl, 0.01M Tris-HCl pH=7.2

Layer over a linear sucrose gradient

60.000 × g, 180 min

Nuclear membrane banded in region between 1.18-1.21 g cm^{-3}

Remove by a cannula and diluite to 0.2M sucrose

110.000 × g, 120 min

Residue: NUCLEAR MEMBRANE

190.000 × g, 24 h.

Supernatant: chromatin proteins

Dialysis against 5M urea, 0.01M Tris HCl pH = 8.3

Ion exchange chromatography QAE Sephadex A-50

- NON-HISTONE PROTEINS ← 3M NaCl, 5M urea, 0.01M Tris-HCl pH=8.3
- HISTONE

Residue: DNA and a small amount of contaminating protein (notably histones)

Suspend in 2M NaCl and 0.01M sodium citrate

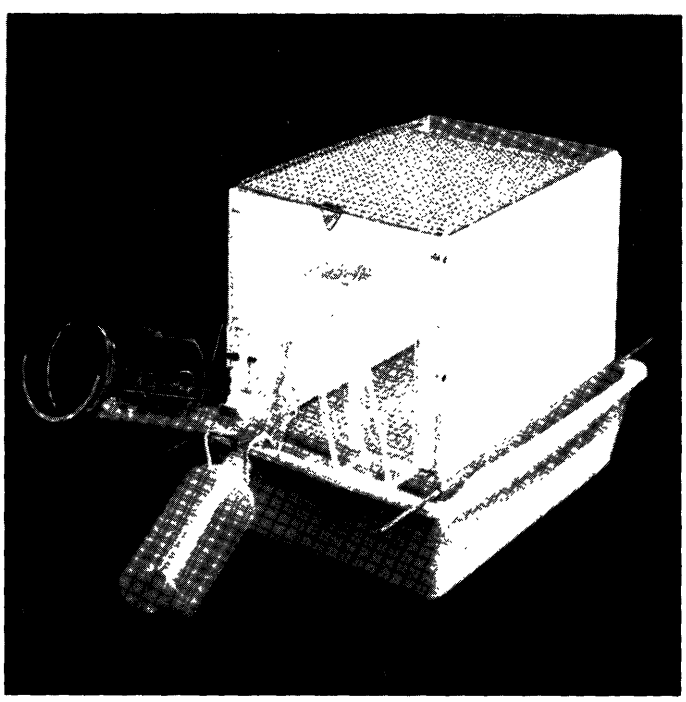
Gel filtration on Sephadex G-75

PURIFIED DNA

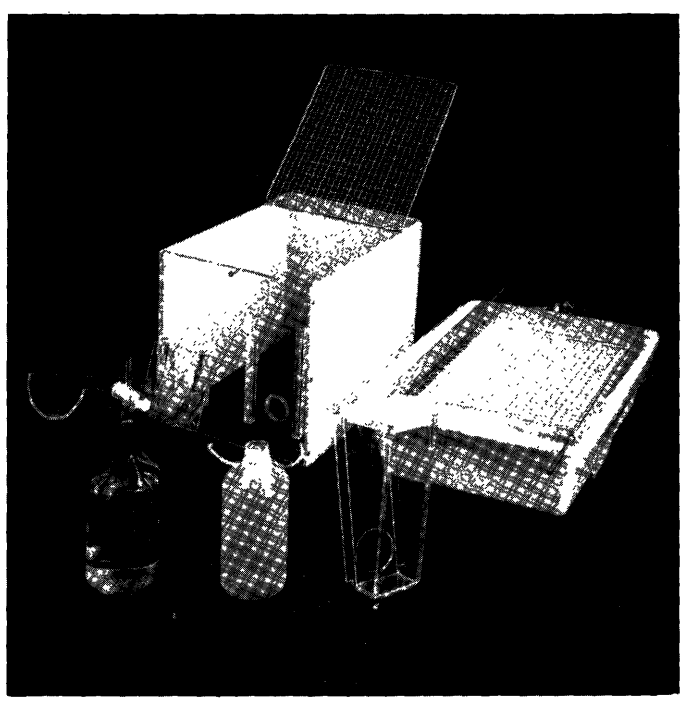
Figure 2

Cold room radiochemical facility

Stabularium



Modular cage



Parts of modular cage

Figure 3

preparation of ^{48}V from Ti target: excitation function

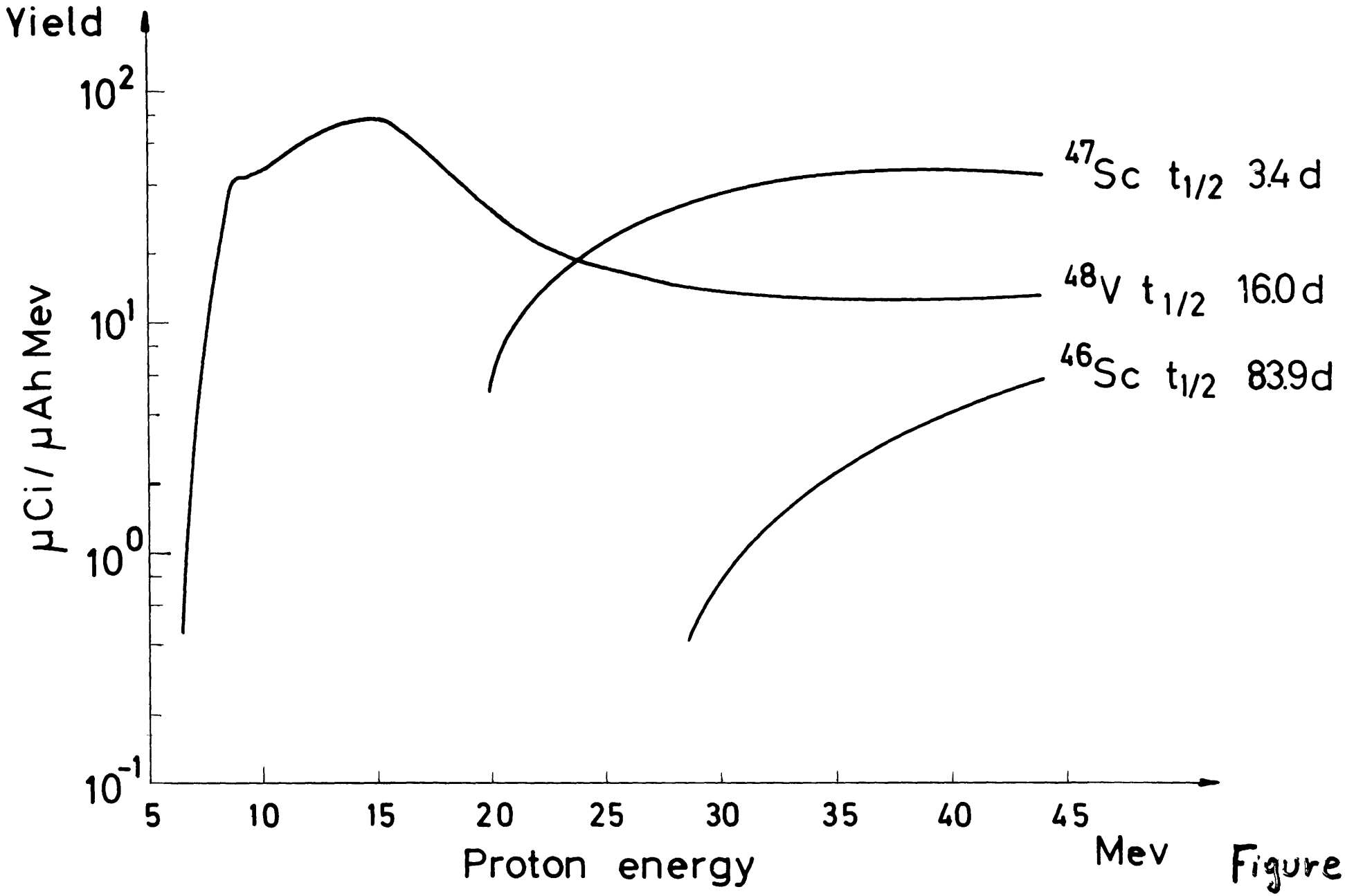


Figure 4

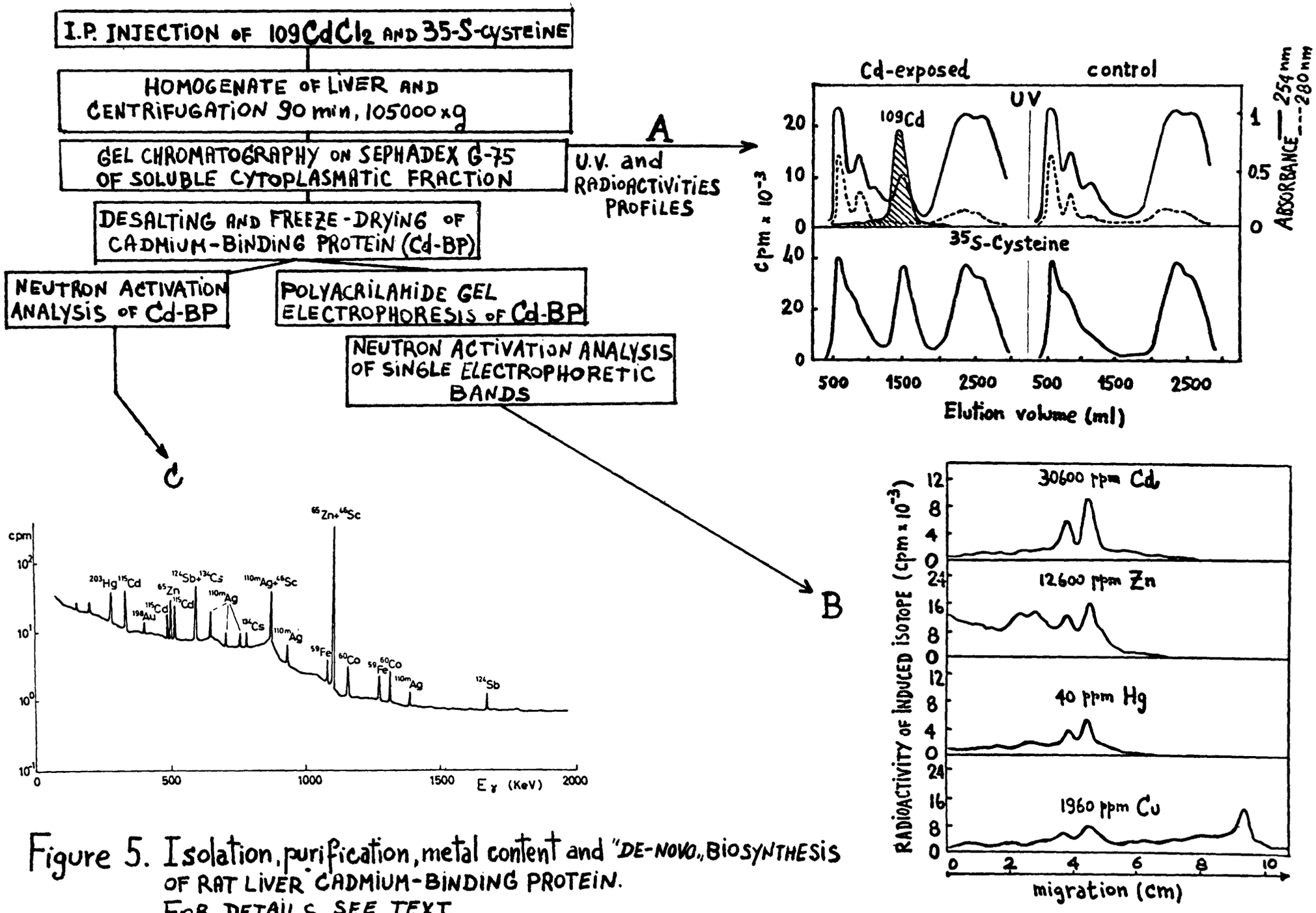


Figure 5. Isolation, purification, metal content and "DE-NOVO" BIOSYNTHESIS OF RAT LIVER CADMIUM-BINDING PROTEIN.
FOR DETAILS, SEE TEXT.

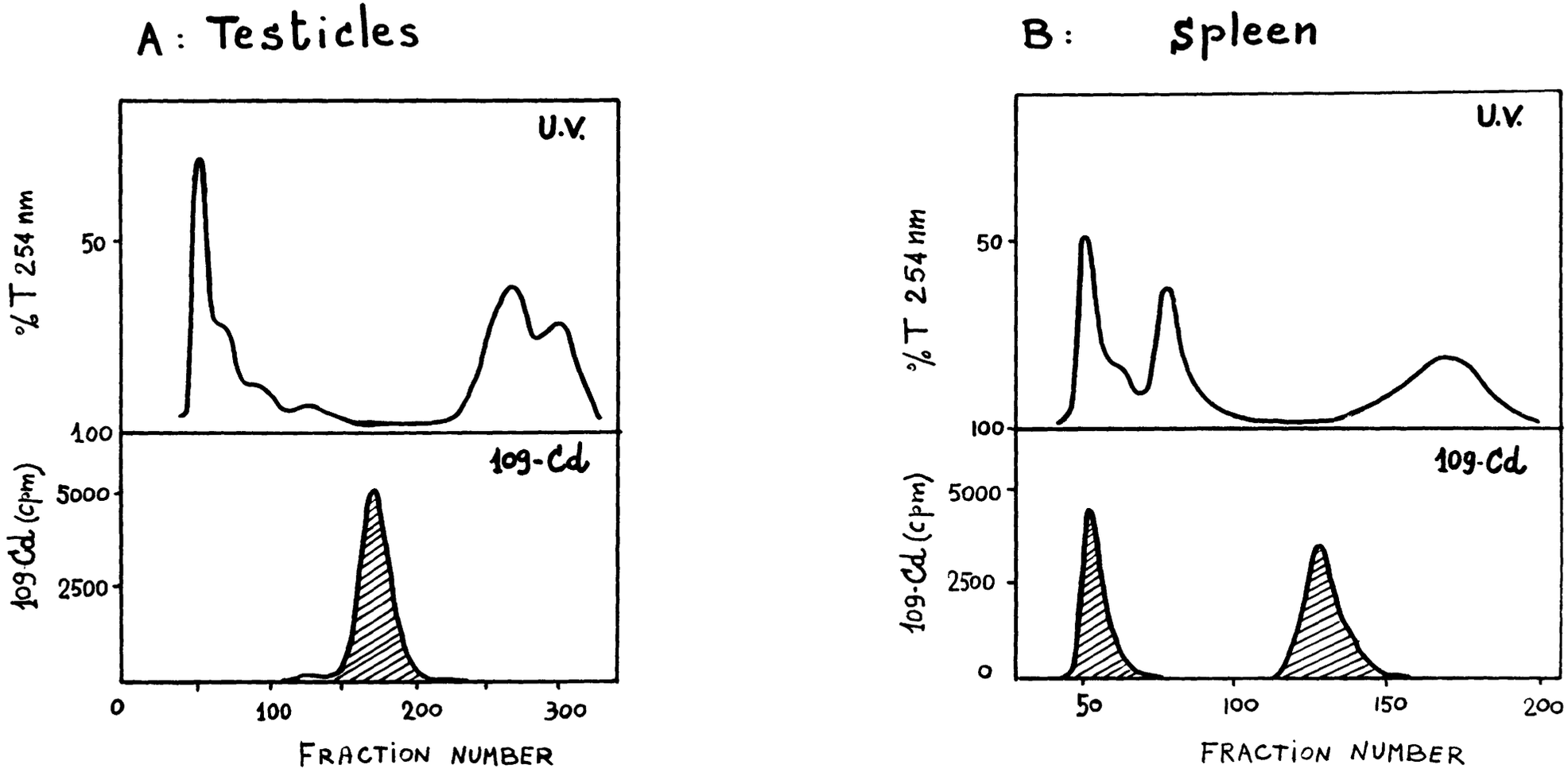


Figure 6. Sephadex G-75 chromatography of the soluble fraction of testicles (A) and spleen (B) of 109-Cd treated rats. A: column 90 × 5 cm. Tris-HCl buffer, pH=8.6 Fractions of 8ml. were collected. B: column 80 × 5cm. Tris-HCl 10mM buffer, pH=8.2 Fractions of 10ml. were collected. Rats were injected i.p. with 109-Cd Cl_2 carrier-free.

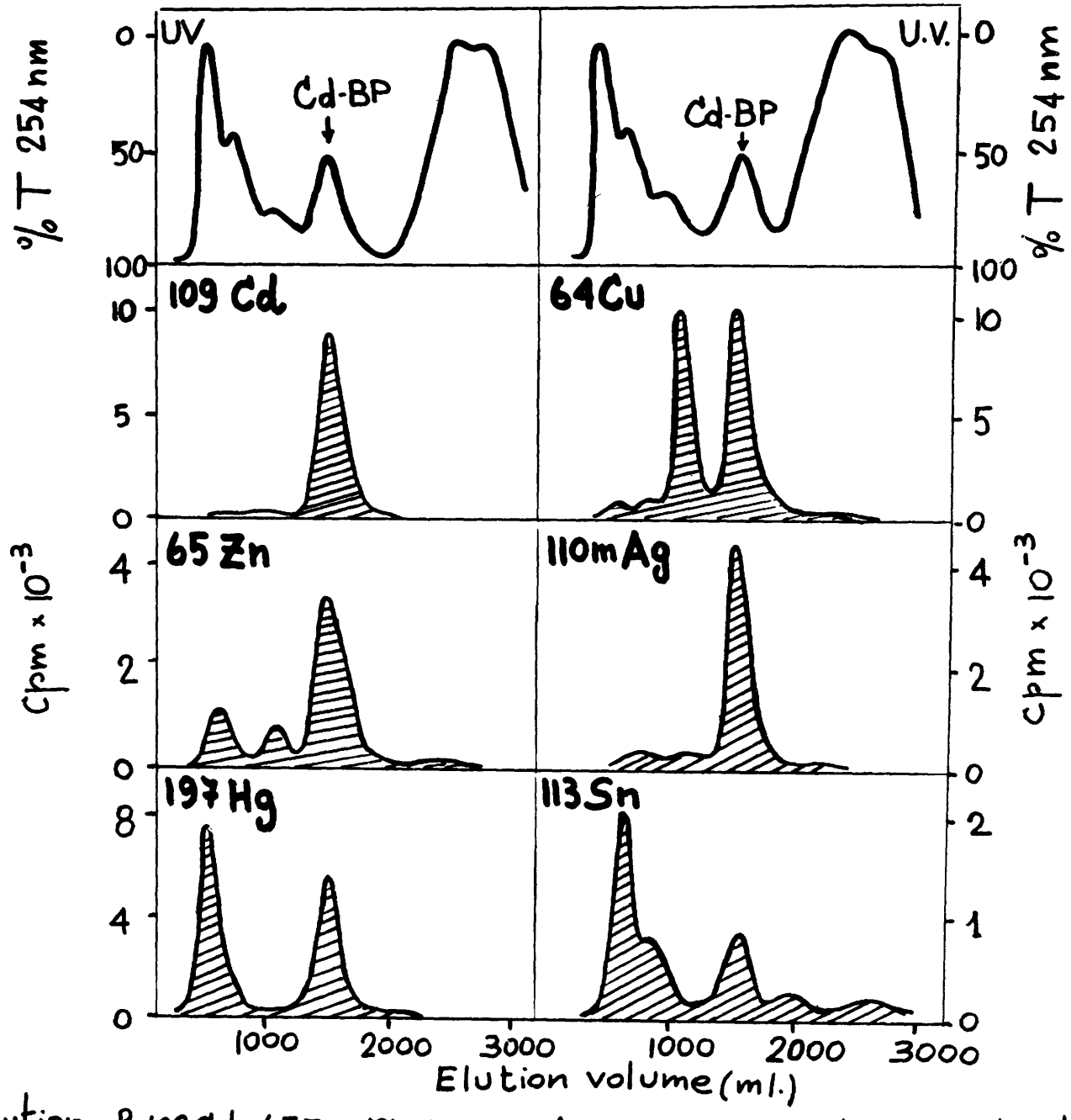


Figure 7. Distribution of ^{109}Cd, ^{65}Zn, ^{197}Hg, ^{110m}Ag and ^{113}Sn in Cd exposed rat liver soluble fraction after Sephadex G-75 chromatography.

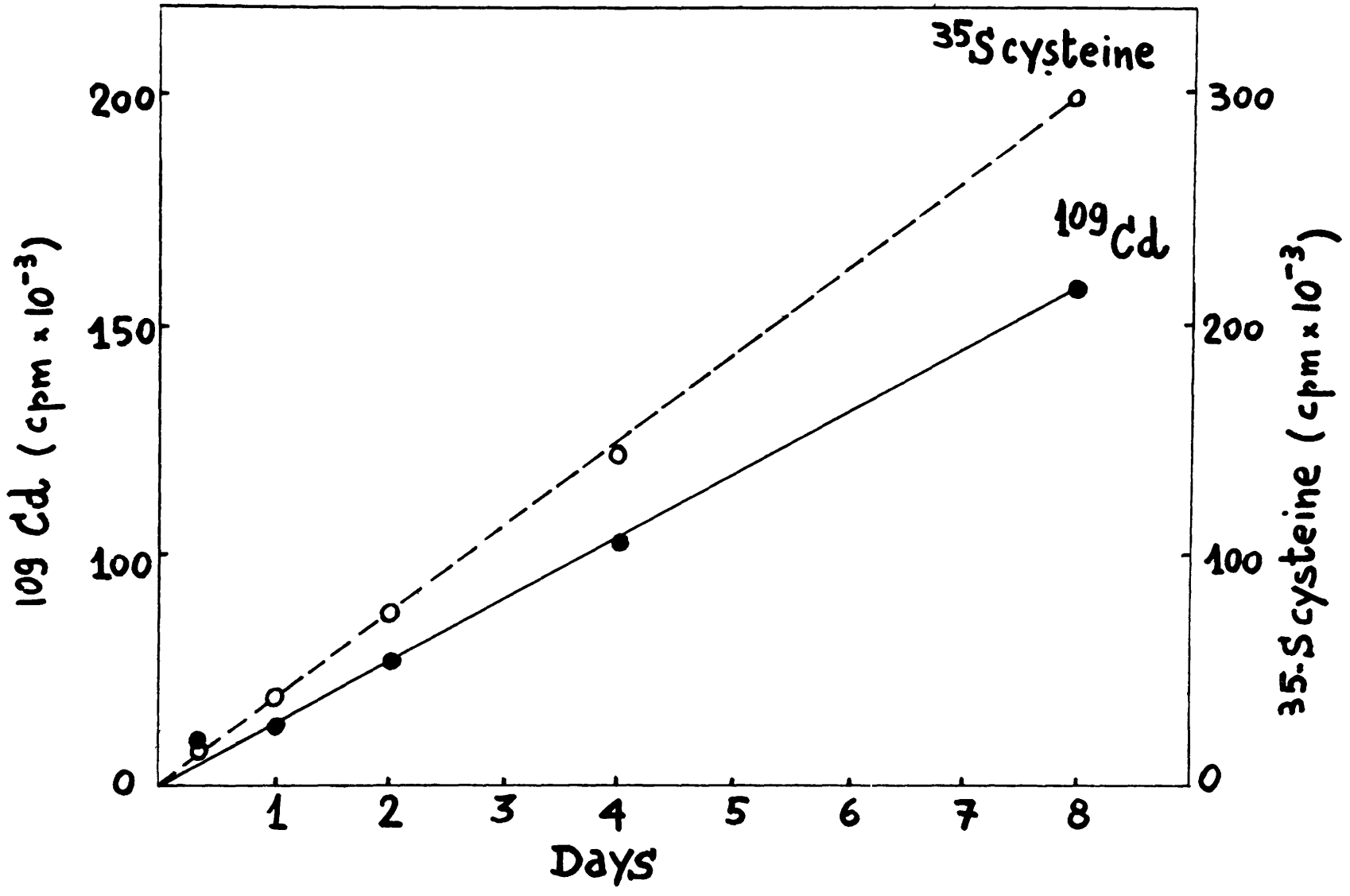


Figure 8. The biosynthesis of cadmium-binding protein as a consequence of the daily accumulation in rat liver. 250 mg of 109-Cd labelled cadmium were injected i.p. for 8 days

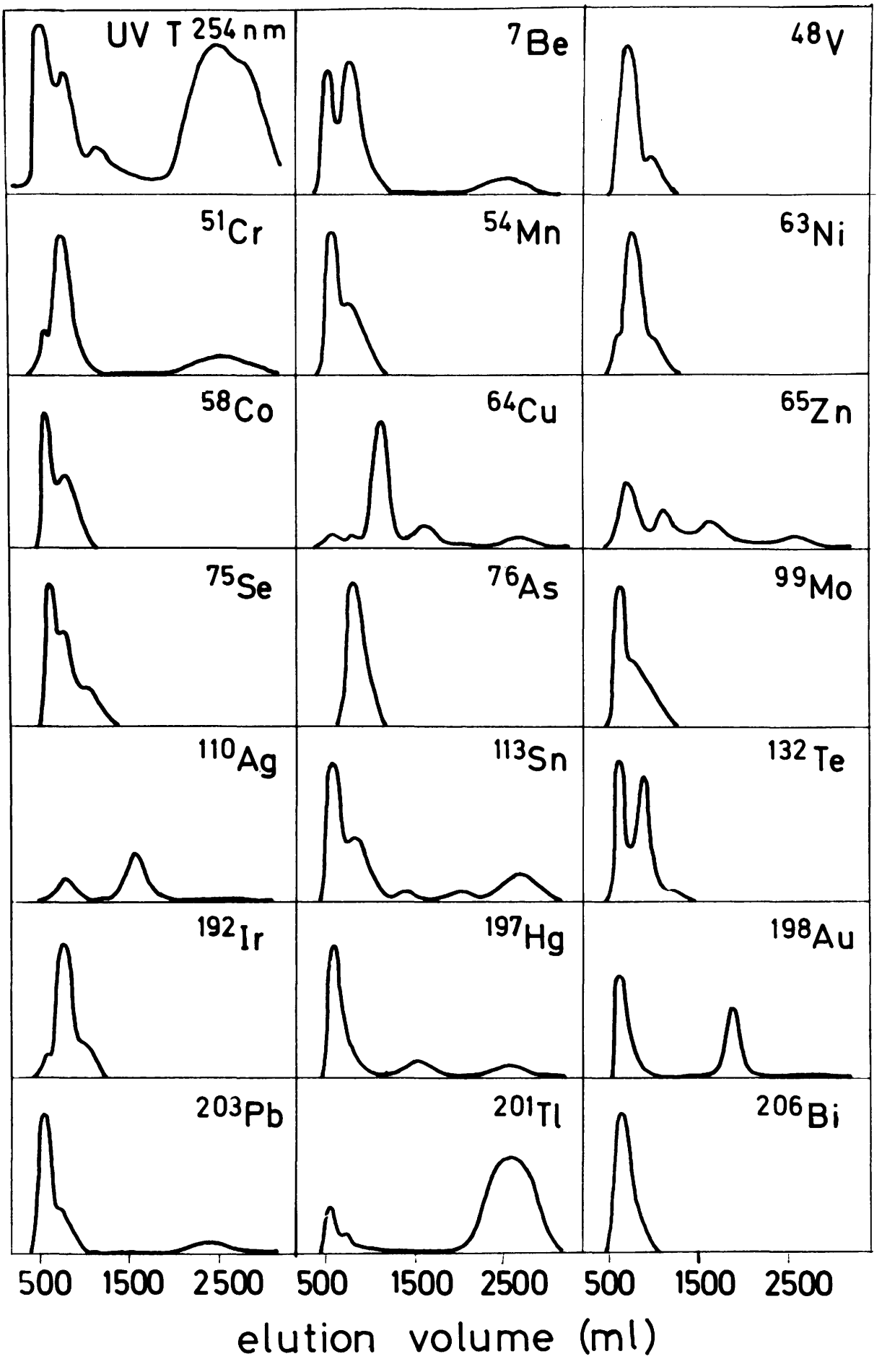


Figure 9. Distribution of radiotracers in soluble fraction from rat liver. UV, Ultraviolet.

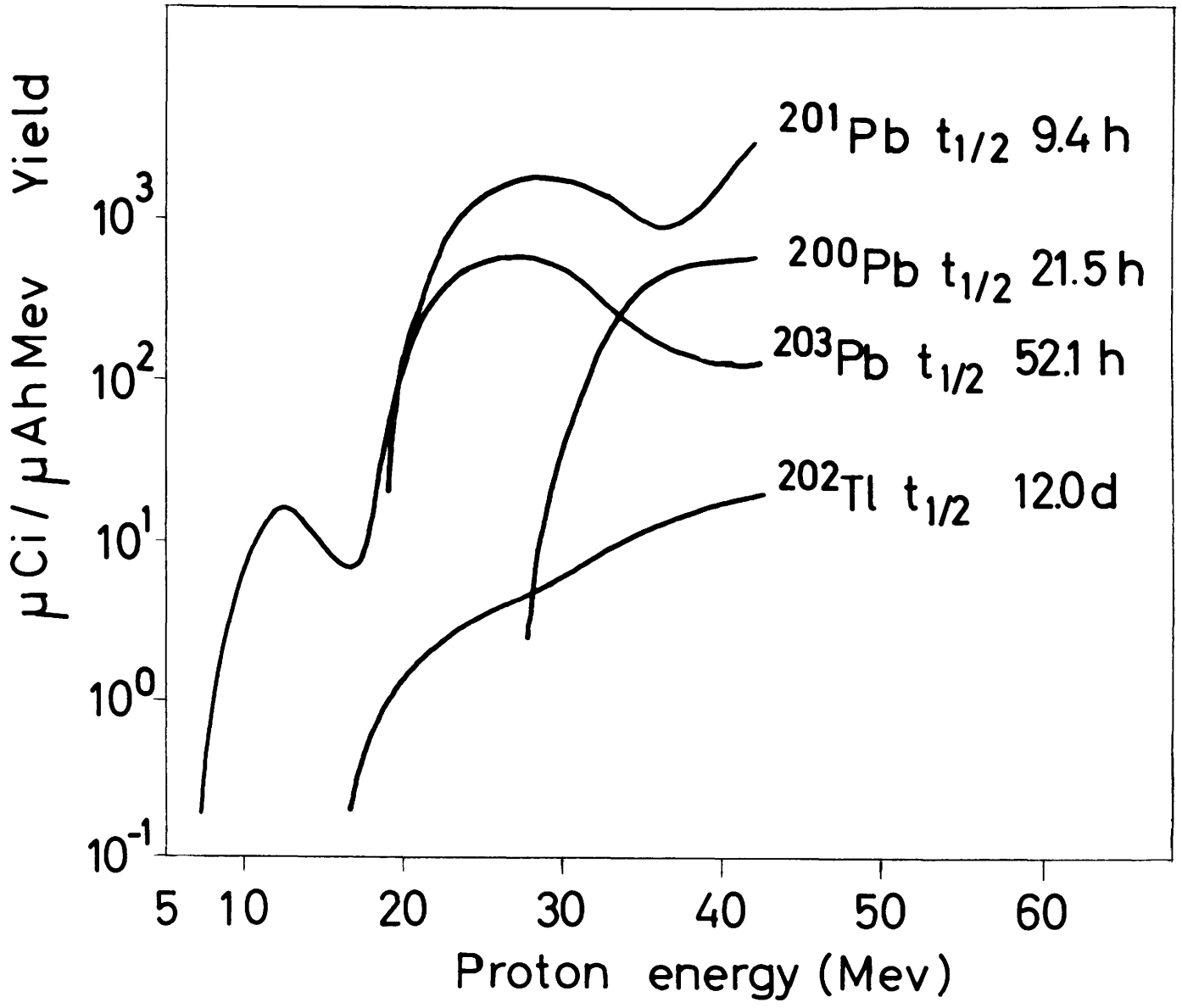


Figure 10. Preparation of 203Pb from thallium target: yield of 201Pb, 200Pb, 203Pb and 202Tl vs. proton energy

	TOTAL BLOOD	KIDNEYS	LIVER	LUNG	SPLEEN	TESTICLES	HEART
% of the dose	3.59 ± 0.28	8.13 ± 0.61	4.37 ± 0.29	0.17 ± 0.02	0.09 ± 0.01	0.03 ± 0.002	0.02 ± 0.002
Mg of Pb	0.65	1.46	0.79	0.03	0.02	0.006	0.004
Mg of Pb/ gm of tissue	0.055	1.25	0.175	0.027	0.058	0.003	0.003

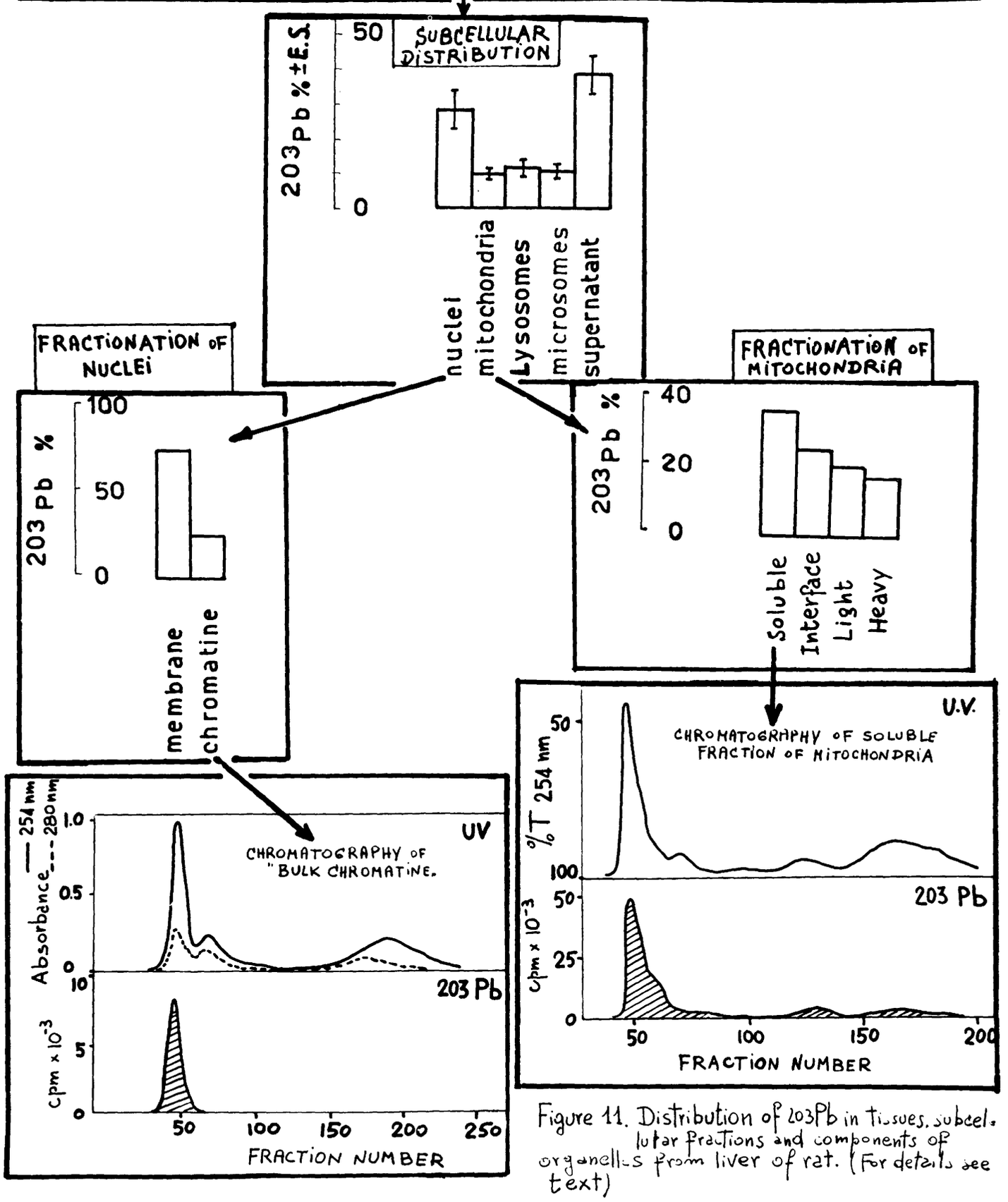


Figure 11. Distribution of 203Pb in tissues, subcellular fractions and components of organelles from liver of rat. (For details see text)

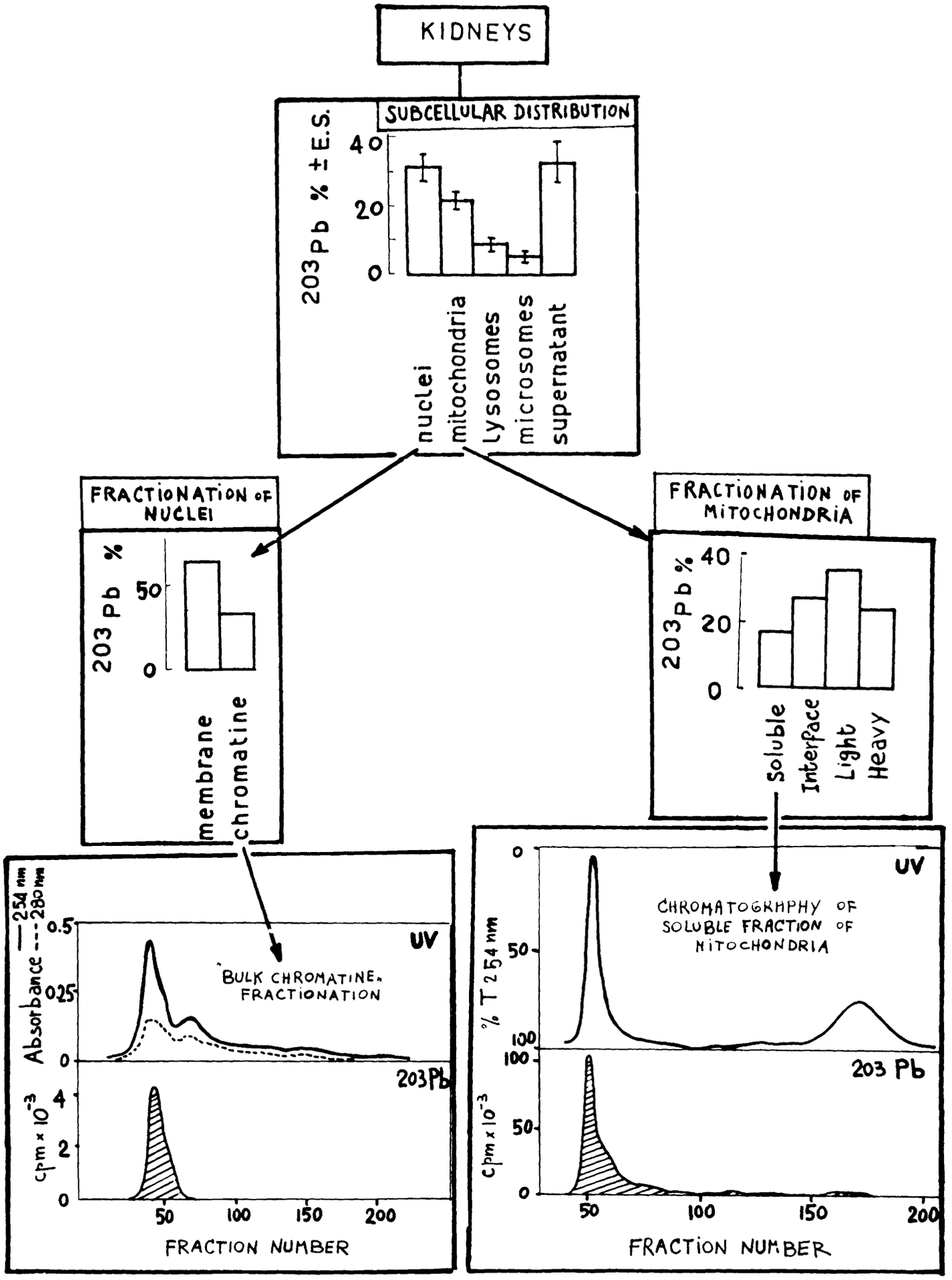


Figure 12. Subcellular distribution of 203Pb in rat kidneys and distribution of radiolead in nuclear and mitochondrial components. (For details see text)

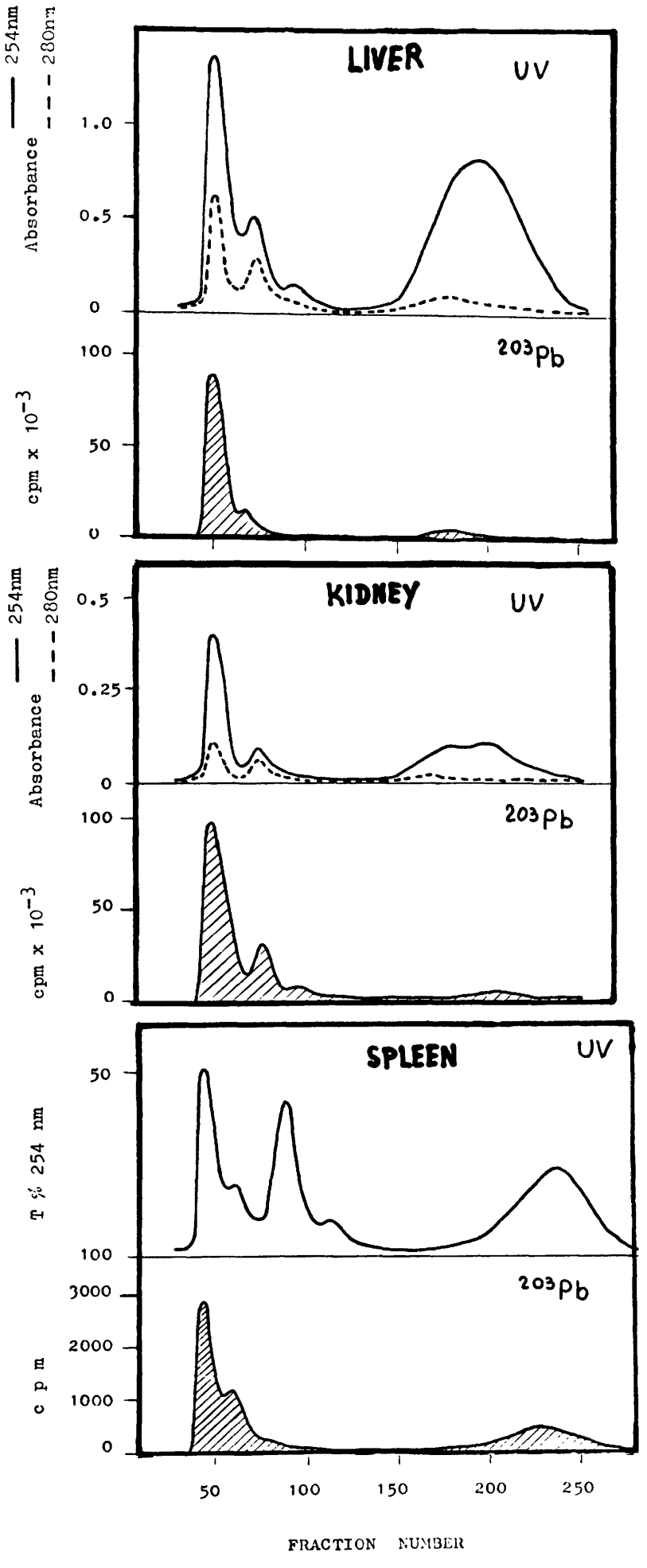


Figure 13. Distribution of 203Pb in the soluble fractions of rat liver, kidney and spleen.