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SELENOPROTEINS AND SELECTED PROTEIN-BOUND TRACE ELEMENTS IN BOVINE KIDNEY CYTOSOL FRACTION

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Dedication

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ABSTRACT

In 1957 Se was shown to be essential for mammals and birds. It was discovered in 1973 that Se is an integral part of glutathione peroxidase (GSH-Px), an enzyme that metabolizes hydroperoxides and protects cells from oxidative damage. Recent studies have indicated that Se may also serve as an anticarcinogenic agent. A few selenoproteins have been identified in various animal organs but their functions have not yet been established. The role of Se in kidneys is unknown. Kidneys contain the highest level of Se among organs; the majority of this Se is not associated with GSH-Px activity.

A combination of bioanalytical and instrumental neutron activation analysis (INAA) methods has been developed in this work to study selenoproteins and other protein-bound trace elements in the cytosol fraction of bovine kidneys. Differential centrifugation, dialysis, ammonium sulfate precipitation, organic solvent precipitation, assays of enzymatic activities, ion exchange chromatography, gel permeation chromatography, polyacrylamide gel electrophoresis and isoelectric focusing have been applied for separation, purification and characterization of proteins. An INAA method has been developed for the simultaneous multielement determination of total inorganic content in the separated samples. Internal and external quality assessment steps have been followed to ensure the quality of results obtained. The INAA method developed provides very good precision and accuracy.

The existence of three novel Se-containing proteins in the cytosol fraction is reported. These are: (i) Selenoprotein A1 (MW of 71 kDa, pI of 5.5); (ii) Selenoprotein P1 (MW of 62 kDa, pI of 5.4); and (iii) Selenoprotein P2 (MW of 48 kDa, pI of 4.9).

The behavior of 18 elements in bovine kidney cytosol has been examined. Results show > 90% of Cu, Fe, Mo, S, Se, V and Zn, and about 70% of Ca, Mg and Mn as bound to macromolecules. Arsenic, Cu, I, Mn, V and Zn have been further investigated using ion-exchange chromatography on DEAE-Sepharose. Results indicate the possible existence of several novel metalloproteins.

ABBREVIATIONS AND SYMBOLS USED

α	alpha-ray
AAS	atomic absorption spectrometry
Ac	acetate
AES	atomic emission spectrometry
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
β	beta-ray
BCA	bicinchoninic acid
BSA	bovine serum albumin
cm	centimetre
CINAA	cyclic instrumental neutron activation analysis
CR	cadmium ratio
CRM	certified reference material
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DUSR	Dalhousie University SLOWPOKE-2 Reactor
E	energy
EAAS	electrothermal atomization absorption spectrometry
EDTA	ethylenediaminetetraacetic acid
FAAS	flame atomic absorption spectrometry
FPLC	fast protein liquid chromatography
FWHM	full width at half maximum
g	gram
γ	gamma-ray
GC	gas chromatography
GSH	reduced glutathione
GSH-Px	glutathione peroxidase
GSSG-R	glutathione reductase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography

ICP	inductively coupled plasma
IEF	isoelectric focusing
INAA	instrumental neutron activation analysis
IR	infrared
IUB	International Union of Biochemistry
keV	kilo electron Volt
kDa	kiloDalton
kg	kilogram
L_C	decision limit
L_D	detection limit
L_Q	determination limit
LC	liquid chromatography
μ_B	number of background counts under a peak
mL	millilitre
µL	microlitre
µg	microgram
min	minute
MS	mass spectrometry
MT	metallothionein
MWCO	molecular weight cut-off
n	neutron
NAA	neutron activation analysis
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
ng	nanogram
NIST	National Institute of Standards and Technology
NRC	National Research Council
p	proton
PAGE	polyacrylamide gel electrophoresis
pg	picogram
pI	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
ppb	parts per billion
ppm	parts per million
RM	reference material
rpm	revolution per minute
RSA	relative specific activity
RSD	relative standard deviation

s	second
SD	standard deviation
SDS	sodium dodecyl sulfate
SLOWPOKE	Safe Low Power Critical Experiment
$t_{1/2}$	half-life of a nuclide
t_c	counting time
t_d	decay time
t_i	irradiation time
TCA	trichloroacetic acid
TGA	thymine-guanine-adenine triplet
$TMSe^+$	trimethylselenonium ion
Tris	Tris-(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
u	unit of enzymatic activity
UGA	uracil-guanine-adenine triplet
UV	ultraviolet
WMD	white muscle disease

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1. INTRODUCTION

Until relatively recently the influence of trace elements on biological systems was strictly categorized as either essential or toxic; however, this distinct division is gradually disappearing. Both laboratory experiments and epidemiological studies suggest that most elements are toxic when present in high amounts and several elements which were previously well known only for their toxicity at high levels now appear essential at much lower concentrations [1]. This new approach can be illustrated by changes in understanding of the role of trace elements such as B, Si, V, Ni and As which were categorized as non-essential at low levels. Their nutritional importance for animals [2] implies their possible importance for humans as well but extensive research is required to recognize biological functions of these elements.

In the studies on trace elements in biological samples the attention has been mainly focussed on the determination of the total content of the elements. The data on "total" element concentrations are useful in many investigations. For example, the determination of total elemental content in toxicology helps to understand how a toxic exposure to one or several elements influences the content of other elements in organs [3], [4], [5]. In biomedical research, investigations of various health conditions and diseases are carried out on the basis of the changes in the total elemental content of a body or of a particular organ [6], [7], [8]. In

epidemiological studies, elemental analysis of specimens such as blood, urine and hair is used to monitor an inadequate or elevated intake of an element by humans [9].

However, with increasing knowledge on the metabolism and biological functions of trace elements it became evident that there is a necessity to quantitatively determine different chemical forms of an element and just not its total amount. Some elements are known to exist as integral parts of proteins and enzymes. The total amount of some of these metalloproteins, however, cannot account for the total amount of the undialysed element found in a sample. These observations indicate that the remaining metal may be bound to proteins which remain to be discovered.

Selenium is one of these trace elements whose essential role in living organisms has been recognized relatively recently. Although it was found in 1950s to partially ameliorate the symptoms of vitamin E deficiency in experimental animals [10], both the metabolic basis for its nutritional functions and its significance in human health remained unclear until 1970s. The first step in the understanding of its nutritional role was made when Se was found to be an essential component of glutathione peroxidase, an enzyme that metabolizes peroxides including lipid peroxides [11]. This discovery showed that Se is involved, together with vitamin E and some other components of the cell, in protecting polyunsaturated membrane lipids from the oxidative degradation that would cause dysfunction of the membrane. The next important breakthrough took

place in 1961 when in China it was demonstrated that a clinical condition in humans, a cardiomyopathy called Keshan disease, was associated with Se deficiency [12].

Animals deficient in Se generally exhibit poor growth, infertility, impaired neutrophil function and increased susceptibility to infection. Several diseases, including liver necrosis in rats, exudative diathesis in chicks, mulberry heart in swine and some forms of muscular dystrophy in lambs were shown to be prevented by either vitamin E or Se supplementation [13], [14], [15]. The distinct and unique role of Se has been demonstrated in 1969 by Thompson and Scott [16] in their studies on chicks on a diet deficient in Se but not in vitamin E; the birds still developed a degeneration of the pancreas. Since then some other beneficial properties of Se have been investigated which included its protective role in cardiovascular diseases and anticarcinogenic effects in tumors of experimental animals [17], [18]. Most recent progress in understanding the Se essentiality links low Se status with occurrence of several AIDS-related conditions such as myopathy, cardiomyopathy and immune dysfunctions [19]. This postulate is supported by findings that Se deficiency is common in HIV positive patients as documented by low plasma and red blood cell levels of Se, diminished activity of glutathione peroxidase and low Se levels in heart [20]. Selenium supplementation apparently causes symptomatic improvements and possibly slows the course of HIV-induced disease [21], [22].

When the level of Se in living organisms exceeds the safe range the

element becomes highly toxic. In mammals, an excess of Se is reported to be teratogenic, cataractogenic, cariogenic, hepatotoxic and neurotoxic causing retarded growth and muscular weakness [23], [24]. Experiments on laboratory animals revealed that the poisonous dose of Se depends on its chemical form, presence of other compounds in diet and the time of supplementation [25]. The toxicity of Se can be inhibited by vitamin E and high protein content in diet and is demonstrated to be altered by interaction with heavy metals [26].

Diet is the major source of Se. The element is present in foods and feedstuffs almost exclusively in organic compounds including selenomethionine, Se-methylselenomethionine, selenocystine, and selenocysteine [27]. Selenite is also an important dietary form of Se because it is used to supplement Se in Se-deficient diet [28], [29]. The metabolism of the different forms of Se varies considerably and is still not well understood. Selenomethionine follows the metabolic pathways of methionine; selenocysteine appears to be catabolized rapidly to release its Se moiety in the form of selenide; selenate and selenite are reduced to selenide. Urine is the predominant route of excretion of Se in the form of trimethylselenonium ion [30].

Still little is known and much is speculated about the biochemistry of Se. Chemically it closely resembles sulfur; in living organisms it is incorporated in at least seven S-analogues of amino acids. There are, however, two main differences between those two elements that determine their distinct metabolism in cells. The first is that Se-species tend to undergo reduction to Se^{2-} while S-

analogues tend to oxidize to S^{6+}. Secondly, at physiological pH the selenohydryl groups of selenols are mostly dissociated whereas sulfhydryl groups of thiols are protonated. These dissimilarities may account for some toxic effects of Se when it randomly substitutes S in proteins.

Selenium is present in various amounts in all animal organs, primarily in kidney, liver and testes and in the cell it is located predominantly in the nucleus and cytosol fractions [31]. The fact that only a part of this Se is associated with glutathione peroxidase activity [32] gave impetus for the search for new biologically important Se molecules. Several other selenoproteins were discovered along with the findings that with insufficient dietary Se intake the supply of Se to these proteins had priority over that to glutathione peroxidase [32]. Among the identified selenoproteins whose plausible functions in the living organism have been postulated are: (i) a 10-kDa protein in heart and muscle of Se-adequate lambs [33]; (ii) a 15-17-kDa protein in rat sperm tail [34]; (iii) a 14-kDa fatty acid-binding protein in mouse liver [35]; (iv) a 57-kDa glycoprotein P in rat plasma [36]; and (v) a 27.8-kDa type I iodothyronine 5'-deiodinase in rat thyroid, kidney and liver [37], [38]. In all these proteins, including glutathione peroxidase, Se is present in the polypeptide backbone in the form of the amino acid selenocysteine. Recent studies showed that the element was incorporated specifically into selenocysteinyl residue through a cotranslational process involving the UGA termination codon, recognizing this way selenocysteine as a twenty first amino acid in terms of protein biosynthesis [39].

The term "selenoprotein", meaning Se-containing protein, has been used in majority of publications but not always clearly defined. The true selenoprotein, of course, is a protein in which the element is incorporated in a form of selenocysteine. The TGA codon is found in the gene encoding this protein. Not all researchers in this field are interested in molecular biology aspect. Some of them use the term selenoprotein towards the molecules in which Se can still be detected despite a rigorous extraction and separation procedures [40], [41]. Indeed, if heating or breaking the protein into subunits does not liberate Se from it then the element cannot be weakly, loosely or randomly bound to this molecule. Instead, it seems to be specifically incorporated into the protein structure and consequently possess a defined and unique function in the living organism. In cases where the nature of binding of Se to the protein is not investigated by researchers, the terms "selenoprotein" and "Se-containing protein" are generally used synonymously.

Kidneys play a major role in physiological homeostasis by regulating the excretion and absorption of numerous substrates from plasma. They are especially important in maintaining suitable concentrations of trace elements in the body. It is well-established that most biochemical reactions require the presence of trace elements within a precise and narrow range of concentrations. Therefore, the proper understanding of these mechanisms would not be possible without investigating the total elemental content as well as the presence of particular protein-bound species. Kidneys have been known for a long time to contain the

highest level of Se among other body tissues and fluids [42]. Behne and Wolters [32] reported 1.45 ± 0.25 ppm Se in rat kidney. At the same time, the highest percentage of Se bound to glutathione peroxidase was found in erythrocytes (82%) and liver (63%) but not in kidneys (11%). Similar results were later obtained by Hawkes *et al.* [31] who investigated tissue distribution of selenocysteine-containing proteins in rats. Selenium concentration in kidneys was 1.6 ppm, 11% of which was accounted for by glutathione peroxidase activity. Although these numbers may vary from species to species, the above findings should urge scientists to look for the existence of other Se-containing macromolecules in kidneys. However, not much is reported in the literature regarding this organ. While several selenoproteins with suggested functions were purified from liver, heart, muscle, testes, sperm and/or blood, the presence of only a few in kidneys was reported [43], [41]. Only one with a molecular weight of 75 kDa was detected in the cytosol fraction [43]. This protein was not purified further and its biological function is not known.

Majority of the studies on selenoproteins involve injecting animals with trace amounts of ^{75}Se, a γ-emitting isotope of Se [44], [41] while others use isotopic labelling of Se in the body with [^{75}Se]selenite through diet [43]. The Se-containing proteins are then isolated and fractionated and the activity of the radiotracer determined quantitatively. However, if the radioisotope is administered in elevated amounts, random substitution of Se for S or random binding of Se to proteins may take place. This may lead to erroneous high Se levels and, moreover, to the

detection of Se-containing proteins that otherwise would not bind Se [29], [45], [46]. On the other hand, if the time of the administeration of the radioisotope is not long enough to establish equilibrium, the obtained Se content will be lower than it really is. Another drawback of using a radiotracer method is its limited applicability to humans and the hazards associated with handling radioactive samples. Alternatively, the naturally-occurring selenoproteins can be separated and purified by biochemical techniques followed by the direct measurement of Se. This approach, initiated in our laboratory by Jayawickreme towards Se- and other protein-bound trace elements [47], [48] is being applied and further developed.

Several instrumental methods of detection, *e.g.* atomic absorption spectrometry, fluorometry, photometry, inductively coupled plasma/mass spectrometry, neutron activation and others are capable of measuring low levels of Se in various biological samples. Their characteristics differ in various aspects such as instrument sensitivity, accuracy and precision of results, sample preparation, requirements for sample size and type, and accessibility for a routine application. Constant attention is needed to improve accuracy and precision of measurements at very low levels and to eliminate errors arising from contamination.

The application of instrumental neutron activation analysis (INAA) for the determination of Se in biological samples has been reported in the literature [49], [50], [51], [52]. This technique permits measurements of trace amounts of Se in milligram quantities of samples and provides excellent precision, accuracy and

freedom from reagent blanks. Six naturally-occurring stable isotopes of Se are known. These isotopes (percent abundances) are: ^{80}Se (49.8%), ^{78}Se (23.5%), ^{82}Se (9.2%), ^{76}Se (9.0%), ^{77}Se (7.6%), and ^{74}Se (0.87%) [53]. At least seven radioisotopes (nuclides) may be produced by neutron activation. Among them, ^{75}Se, ^{77m}Se and ^{81}Se can be used for the quantitative determination of Se by INAA. The ^{75}Se nuclide has proven to be very useful for radiotracer experiments due to its long half-life (120 days). However, ^{75}Se is not particularly suitable for routine measurement purposes by INAA in a large number of samples due to the long irradiation, decay and counting times required. The ^{81}Se nuclide does not provide adequate sensitivity. The use of ^{77m}Se (half-life = 17.4 s), on the other hand, allows for a very rapid analysis of a large number of samples. This nuclide has been employed in our laboratory to investigate Se-proteins in biological materials and will also be used in this thesis.

A well-planned biological trace element study requires a broad, multidisciplinary approach. It involves an understanding of the biological basis of the problem and development of appropriate bioanalytical separation methods and elemental detection methods. Identification of sources of errors, especially those arising from contamination of the samples and improper use of bioanalytical techniques, is also of a great importance.

The main objective of this work is to investigate Se-containing proteins occurring naturally in bovine kidney cytosol fraction. In order to achieve this goal,

a combination of biochemical methods for protein separation, purification and characterization will be developed and applied. These methods will include: differential centrifugation, dialysis, ammonium sulfate precipitation, organic solvent precipitation, anion exchange chromatography, gel permeation chromatography, polyacrylamide gel electrophoresis in denaturing and nondenaturing buffer systems and isoelectric focusing. The purities, in terms of cross-contaminations, of cytosol and other subcellular fractions will be evaluated by assaying activities of four marker enzymes. Although this test should precede any further separation studies, it has not been widely reported in the literature.

Instrumental neutron activation will be used as a post-separation method of analysis for the total elemental content of the samples investigated. Internal and external quality assessment steps will be extensively involved in assessing the accuracy and precision of the results generated by INAA. These steps include: (i) selection of appropriate target isotopes and nuclides; (ii) energy calibration of gamma-ray spectrum; (iii) correction for spectral interferences and competing reaction interferences; (iv) measurements of analytical blank; (v) optimization of sample geometry; (vi) analyses of replicate samples under identical conditions; (vii) construction of control charts for comparator standards; and (viii) analyses of standard reference materials.

One of the great features of INAA is its ability to measure multielement concentrations simultaneously. This advantage will be readily exploited to study other trace and minor elements present in bovine kidneys and its cytosol fraction

as well as their possible association with proteins. Arsenic, vanadium, iodine, manganese, copper and zinc will be investigated in more detail. The results obtained will be discussed and presented along with the brief introduction to the present state of knowledge on these trace elements.

2. DEVELOPMENT OF AN INSTRUMENTAL NEUTRON ACTIVATION ANALYSIS METHOD

2.1 INTRODUCTION

The aim of the work presented in this chapter was to develop an INAA method of high precision and accuracy for the measurement of multielement concentrations in bovine kidney. The chapter begins with the outline of the principles of NAA followed by the presentation of examples of the application of this technique to study trace elements in various biological samples. The next section describes several steps of the internal and external quality assessments that were undertaken to provide high level of confidence in the results obtained.

2.2 NEUTRON ACTIVATION ANALYSIS

2.2.1 Principles

Neutron activation analysis (NAA) is an analytical technique that can provide both qualitative and quantitative information on the elemental concentrations of a wide variety of samples. It was developed by Hevesy and Levi [54] in 1936 for the analysis of Dy. In NAA, a sample is irradiated in a flux of neutrons, resulting in the formation of activation products. The gamma-rays originating either from transitions among activated energy states of the nucleus or from decay products are detected. The gamma-ray energies are characteristic of specific nuclides and their intensities (in practice the number of counts detected by a detector) are

proportional to the amounts of various elements in the sample. The constant of this proportionality is determined by several parameters as shown in the following equation [55]:

$$C = m \frac{N}{M} \theta \sigma \phi \frac{\varepsilon \gamma'}{\lambda} (1-e^{-\lambda t_i}) (e^{-\lambda t_d}) (1-e^{-\lambda t_c}) \qquad (2.1)$$

where:

C = number of counts detected;

m = mass of the element (g);

N = Avogadro's Number (mol^{-1});

M = atomic mass of the element ($g\ mol^{-1}$);

θ = abundance of the isotope;

σ = activation cross section of the isotope (cm^2);

Φ = neutron flux ($cm^{-2}\ s^{-1}$);

ε = detector efficiency;

γ' = gamma branching ratio;

$t_{1/2}$ = half-life of the nuclide (s);

$\lambda = (\ln 2)/t_{1/2}$ = decay constant of the nuclide (s^{-1});

t_i = irradiation time (s);

t_d = decay time (s);

t_c = counting time (s).

Although it is possible to apply equation (2.1) directly to obtain elemental concentrations (called "absolute" activation analysis), this is not usually done because of significant uncertainties that may be associated with some of the prameters, *e.g.*, the cross section or the effective flux density. For more reliable results, a "comparator method" is used in which standards containing known amounts of the element of interest are irradiated, often simultaneously, with the samples. A simplification of the equation (2.1) for sample and standard gives:

$$\frac{mass\ of\ Element_{sample}}{mass\ of\ Element_{standard}} = \frac{counts\ of\ Nuclide_{sample}}{counts\ of\ Nuclide_{standard}} \qquad (2.2)$$

The use of this equation assumes that both samples and standards are exposed to the same neutron flux; both are counted using the same detector and geometry; and that the irradiaiton, counting and decay times are the same in both cases. If these conditions are not fulfilled, suitable corrections must be made.

The NAA technique is usually divided into three categories based on whether any chemical separations are carried out, and if so, whether they are done before or after the irradiation. If no chemical treatment is done, the process is called instrumental neutron activation analysis (INAA). If chemical separations are done after irradiation to remove interferences and/or to concentrate the element of interest, the technique is called radiochemical neutron activation analysis (RNAA). If preirradiation chemical separations are employed, the procedure is then called preconcentration neutron activation analysis (PNAA).

The NAA technique is capable of simultaneous multielement determination. The irradiation of a sample induces activity in many isotopes and the use of a multichannel analyzer in conjunction with a high resolution detector allows one to obtain information on many elements. A major advantage of NAA is that it can be made highly sensitive. There are several adjustable experimental parameters, shown in equation (2.1) which can be manipulated to maximize sensitivity for the desired element. These include the type, energy and flux of neutrons. The time of irradiation can be varied resulting in the appropriate selection of elements with either short- or long-lived nuclides. Other advantages of NAA include rapidity, particularly when the analysis is based on short-lived nuclides. In case of INAA, its nondestructive nature offers the advantage of retaining the sample for further analysis by other techniques. The INAA technique is also free of reagent contamination problems. Since most applications of NAA require minimum sample preparation and manipulation, it is easier to obtain reliable results.

2.2.2 Selected Applications of NAA

Neutron activation analysis is being widely applied to study biological materials for their elemental content. Biological materials are well suited to NAA because their major elements (H, C, N and O) are not significantly activated during irradiation. However, they may contain high levels of Na, Cl and Br which can produce high activity. Pre- or post-irradiation chemical separations are applied to the analysis of such samples. Selected applications of NAA to determine elements

of interest in this work are given below.

Concentrations of ten elements, namely Cd, Co, Cr, Fe, Mo, Ni, Se, Ti, V and Zn were determined in biological fluids, human blood serum and market milk using a PNAA method by Lavi and Alfassi [52]. The method enabled them to determine the ng levels of the tracers by eliminating interfering elements such as Na, Br, Cl and K. Radiochemical NAA was used by Garg *et al.* [56] to analyze milk and cancerous breast tissue for Co, Fe, Sb, Se and Zn. Because of the low levels of these elements, it was necessary to adopt radiochemical procedures for their measurements with high precision and accuracy.

Concentrations of As, Cd, Cr, Co, Pb, Sb, Se and Zn in cancerous lung tissue of deceased smelter workers were determined by Gerhardsson and Nordberg [57] using PNAA. The levels of all elements except for Se and Zn were elevated compared to those in healthy subjects. The purpose of these experiments was to investigate the correlation between the level of various trace elements and the occurrence of lung cancer in the exposed persons.

Instrumental NAA was applied to study the effects of diet, age and gender on the mineral composition of bones in monkeys [58]. The levels of Br, Ca, Cu, Mg, Mn, Na, Se, V and Zn were determined. Sodium and Mn showed significant diet-related effects and Ca level was found to change with age and was lower in females than in males.

Vanadium levels in hair and blood of normal and exposed children were determined using INAA and RNAA by Kucera *et al.* [59]. The hair V levels did not

differ significantly but V content in blood was higher in children exposed to V in drinking water compared to that in blood of normal children.

The determination of Se in a wide variety of biological samples *via* its short-lived nuclide ^{77m}Se has been reported in the literature. Selenium can behave as essential or as a toxic element (depending on its level) for humans and animals and its concentration needs to be measured with high accuracy. The use of the short-lived (^{77m}Se, half-life = 17.4 s) rather than the long-lived (^{75}Se, half-life = 120.4 d) nuclide in INAA allows a very rapid analysis of a large number of samples and eliminates a build-up of interfering activity.

Selenium in serum and liver was determined by Blotcky *et al.* [49]. The method gave excellent accuracy. Spectral interferences from ^{23}Ne and ^{19}O were recognized and eliminated. The ^{19}O activity was greatly reduced upon lyophylization of wet liver and raw serum samples; the level of ^{23}Ne in serum originated from ^{23}Na(n,p)^{23}Ne reaction was decreased by using dialysis to remove Na.

McKown and Morris [60] analyzed Se in several animal organs and blood sera. Serum samples were dialysed prior to irradiation in order to eliminate much of Na and Cl. Wet samples were lyophilized prior to analysis in order to reduce ^{19}O activity in the irradiated samples. Selenium in urine was determined by Baskett *et al.* [61]. Because of the high Na content, urine cannot be easily analyzed by INAA. A method of coprecipitation of Se with As to separate Na was developed. Additionally, toenail, whole blood and sera were analyzed as monitors of dietary Se intake. The method was fast and could be applied to epidemiological

studies involving large numbers of samples.

Selenium in food samples was determined using cyclic INAA (CINAA) by McDowell *et al.* [62]. The method provided better precision and detection limits over conventional INAA methods as the peak-to-background ratio improved with increasing number of cycles. The best results were obtained when samples were recycled 4 times. Homogeneity and sampling constants for Se in biological reference materials using CINAA have also been studied [63], [64].

Neutron activation analysis can now be considered a mature field. The principles are well understood. Nevertheless, the field of NAA remains dynamic as researchers continue to develop new methods, look for new applications, improve sample handling and data processing procedures and further enhance the quality (accuracy and precision) of the data generated.

2.3 EXPERIMENTAL

2.3.1 Irradiations

All irradiations were carried out at the Dalhousie University SLOWPOKE-2 Reactor (DUSR) facility. The reactor has three types of pneumatic sites which can be accessed through sample delivery systems: (i) inner site which holds medium-size (7 mL capacity) vials and has a thermal flux of $5x10^{11}$ n cm^{-2} s^{-1} at 10 kW power level; (ii) outer site which holds large-size (27 mL capacity) vials and has a thermal flux of $2.5x10^{11}$ n cm^{-2} s^{-1} at 10 kW; and (iii) cadmium-shielded outer side which holds large-size vials and is used for irradiations with epithermal neutrons

at a flux of 5.2×10^9 n cm^{-2} s^{-1} at 10 kW. The distribution, homogeneity, reproducibility and stability of the neutron flux will be described in Section 2.4.1.1.1.

2.3.2 Gamma-Ray Detection System

Gamma-ray spectra were recorded using an Aptec hyperpure Ge detector. It had a resolution (full-width-at-half-maximum, FWHM) of 2.08 keV at the 1332-keV photopeak of ^{60}Co, peak-to-Compton ratio of 17:1 and a detection efficiency of 3% (relative to a standard NaI(Tl) detector). The detector system was connected to a 4096-channel Nuclear Data ND-66 MCA through a Link 8010 pulse processor and a Link 8020/4 ADC.

2.3.3 Timing Parameters for INAA

The nuclear data of the elements detected in bovine kidneys and reference materials are shown in Table 2.1.

An irradiation time (t_i) of 1 min, decay time (t_d) of 10 s and counting time (t_c) of 1 min were used to detect the short-lived nuclides of Ag, F, Rb and Se.

Concentrations of Al, Ca, Cl, Cu, I, Mg, Mn, Na, S and V were determined *via* their short-lived nuclides. A t_i of 5 min was used for analyzing the Rice Flour and Wheat Flour Standard Reference Materials (SRMs) and the dialysed samples of bovine kidney where the levels of Na and Cl were low and therefore the dead time over the counting period did not exceed 10%. The rest of the samples had

Table 2.1

Nuclear data [53] for elements detected in biological materials by INAA

Element	Nuclide	half-life	E_γ (keV)
Ag	^{110}Ag	24.0 s	658
Al	^{28}Al	2.31 min	1779
As	^{76}As	26.3 h	559
Br	^{80}Br	17. 0 min	617
	^{82}Br	1.47 d	776
Ca	^{49}Ca	8.72 min	3083
Cd	^{115}Cd	2.25 d	335
Cl	^{38}Cl	37.2 min	1642
Co	^{60}Co	5.27 a	1332
Cr	^{51}Cr	27.7 d	320
Cu	^{66}Cu	5.1 min	1039
F	^{20}F	11.0 s	1633
Fe	^{59}Fe	44.6 d	1099
Hg	^{203}Hg	46.8 d	279
I	^{128}I	25.0 min	443
K	^{42}K	12.4 h	1525
Mg	^{27}Mg	9.45 min	843
			1014
Mn	^{56}Mn	2.58 h	846
			1811
Mo	^{99}Mo	66.0 h	140
Na	^{24}Na	15.0 h	1369
Rb	^{86m}Rb	1.04 min	556
S	^{37}S	5.1 min	3102
Sb	^{122}Sb	2.7 d	564
Sc	^{46}Sc	83.85 d	889
Se	^{77m}Se	17.4 s	162
	^{75}Se	120.4 d	265
Sn	^{113}Sn	115.1 d	392
V	^{52}V	3.76 min	1434
W	^{187}W	23.9 h	686
Zn	^{69m}Zn	13.8 h	439
	^{65}Zn	243.8 d	1115

to be irradiated for 1 min because of their high Na and Cl content. The decay and counting times were same in both cases, *i.e.* 1 min and 10 min, respectively. For determining Mn in materials with high Mg and relatively low Mn content, samples were allowed to decay for 1 h and then counted again for ^{56}Mn only.

Nuclides of As, Br, Cd, K, Mo, W and Zn were detected using t_i = 7 h, t_d = 16 h and t_c = 0.5 h. In order to determine the concentrations of Co, Cr, Fe, Hg, Sc, Se and Sn, samples were allowed to decay for 16 d and then counted for 1 h.

2.3.4 Preparation of Samples for Irradiation

Polyethylene vials were purchased from the Olympic Plastics Company, Inc., U.S.A. Dry samples were packed into the vials without any pretreatment. Wet samples were freeze-dried and then the powder transferred to the vials. The liquids were gradually poured into the vials, evaporated under an IR lamp and an additional portion of the aliquot added. The procedure was repeated until the desired volume of sample was transferred. The advantage of this method of liquid sample preparation was that the position of sample inside the vial could be kept fixed and hence the irradiation and counting geometry remained identical. A trimmed vial cap was pushed down the vial to maintain the desired, constant geometry. Vials were then capped and heat-sealed to prevent spilling of the radioactive material during and after irradiation. Two different sample geometries were used throughout these studies: (i) half-full volume of the small polyethylene irradiation vial (1.1 mL capacity), and (ii) a small, defined volume (0.5-cm vial

height), pressed to the bottom of the medium-size polyethylene vial (7 mL capacity) by a trimmed cap.

2.3.5 Comparator Standards

Elemental comparator standards were prepared in a solid form on a sucrose matrix (Koch - Light Laboratories Ltd., England). For most elements, the plasma emission spectroscopy standard solutions of certified purity (usually 99.999%) were obtained from Seigniory Chemical Products Ltd. The initial concentrations of these solutions were 1000 μg mL^{-1} and they were diluted with distilled deionized water (DDW) to desired concentrations. Vials were first filled with sucrose to a certain height; then appropriate amounts of standards in a few μL and a few drops of DDW were added to ensure homogeneity throughout the sucrose. The samples were dried under an IR lamp. Standards for Ca, F, Fe, I and S were prepared from the following solids: $CaSO_4$ from Fisher Scientific Co., NH_4F and $FeNH_4(SO_4)_2$ $12H_2O$ from Aldrich, KI from Mallinckrodt Chemical Works, and $(NH_4)_2SO_4$ from Sigma. Standards in two different geometries were prepared so that samples of both geometries could be analyzed. Five to seven replicates of each standard were analyzed.

2.3.6 Certified Reference Materials

In this study, five reference materials (RMs) and standard reference materials (SRMs) were analyzed. These materials were: Dogfish Liver (NRC RM

DOLT-1) and Dogfish Muscle (NRC RM DORM-1) supplied by the National Research Council (NRC) of Canada; and Bovine Liver (NIST SRM 1577a), Rice Flour (NIST SRM 1568) and Wheat Flour (NIST SRM 1567) provided by the U.S. National Institute of Standards and Technology (NIST). All five materials were analyzed in small polyethylene vials with a half-full-vial geometry. The Bovine Liver SRM was also analyzed in a medium-size vial employing the second type of geometry. The materials were dried according to the procedures recommended by the suppliers. Four replicates of each material were prepared and then run under different experimental conditions depending on the elements of interest.

2.3.7 Interferences

Two types of interferences were encountered over the course of this study: (i) competing reactions which occurred during activation where the same nuclide was produced from different parent isotopes; and (ii) spectral interference where two distinct nuclides had photopeaks of the same or very similar energy. Interferences of the first type took place in the determination of Al, Mg and F and the second one in the measurements of Mn and Hg contents.

The 1779-keV photopeak of ^{28}Al was used to determine aluminum in bovine kidney and reference materials. This nuclide is produced from the stable isotope of aluminum, ^{27}Al, according to the reaction:

$$^{27}Al\ (n,\gamma)\ ^{28}Al \qquad (2.3)$$

However, the same nuclide is also generated from P and Si *via* the following reactions:

$$^{31}P\ (n,\alpha)\ ^{28}Al \qquad (2.4)$$

and

$$^{28}Si\ (n,p)\ ^{28}Al \qquad (2.5)$$

Silicon is generally present in biological materials in very small quantities, therefore the ^{28}Al activity arising from the reaction (2.5) is negligible. Interference from P was evaluated in this work using a combination of INAA and epithermal INAA (EINAA) methods.

Epithermal neutrons are available in the DUSR facility's cadmium-shielded irradiation site. Cadmium is used to absorb neutrons with energies below *ca.* 0.5 eV.

The total ^{28}Al activity generated in reactor flux comes both from ^{27}Al and ^{31}P and can be presented by the following equation:

$$Al_{T1} = Al_{Al1} + Al_{P1} \qquad (2.6)$$

where:

Al_{T1} - total activity after irradiation in reactor flux neutrons;

Al_{Al1} - activity produced from parent isotope ^{27}Al;

Al_{P1} - activity produced from the interfering isotope ^{31}P.

Similarly:

$$AI_{T2} = AI_{Al2} + AI_{P2} \tag{2.7}$$

where the activities come from irradiation in epithermal neutrons.

The cadmium ratio (CR) of a nuclide is an experimentally determined factor that relates the activity of a nuclide arising from activation in the reactor neutron flux with that in the epithermal neutron flux. For Al and P, these ratios are defined as:

$$CR_{Al} = \frac{AI_{Al1}}{AI_{Al2}} \tag{2.8}$$

and

$$CR_{P} = \frac{AI_{P1}}{AI_{P2}} \tag{2.9}$$

And after substitution and rearrangement:

$$AI_{Al1} = \frac{\frac{1}{CR_P} AI_{T1} - AI_{T2}}{\frac{1}{CR_P} - \frac{1}{CR_{Al}}} \tag{2.10}$$

If the CR_P approaches 1 and $CR_{Al} \gg 1$, the following approximation applies:

$$AI_{Al1} = AI_{T1} - AI_{T2} \tag{2.11}$$

Therefore, by irradiating the same sample both in a normal reactor site and a

cadmium-shielded site, ^{28}Al produced only from the ^{27}Al present in a sample can be calculated.

Magnesium was determined *via* ^{27}Mg nuclide using either the 843-keV or 1014-keV photopeak. This nuclide can be produced from ^{26}Mg and ^{27}Al *via* the following two reactions:

$$^{26}Mg\ (n,\gamma)\ ^{27}Mg \qquad (2.12)$$

and

$$^{27}Al\ (n,p)\ ^{27}Mg \qquad (2.13)$$

In this case, the Al interference was eliminated using the same correction method as described for the ^{31}P - ^{28}Al interference.

The ^{20}F nuclide can be produced by the following reactions:

$$^{19}F\ (n,\gamma)\ ^{20}F \qquad (2.14)$$

$$^{23}Na\ (n,\alpha)\ ^{20}F \qquad (2.15)$$

The correction method used to determine Al and Mg could not be used with short-lived nuclides for practical reasons. In this case, the activity of ^{20}F produced from ^{23}Na was first determined. It was done by calculating the ratio of two peaks (the 1369-keV peak of ^{24}Na and the 1633-keV peak of ^{20}F) in the Na comparator standard run under conditions of t_i = 1 min, t_d = 10 s and t_c = 1 min. The same ratio was then measured in the samples analyzed; if the ratio was greater than

that determined in the comparator standard, the difference in the number of counts was considered to come from F present in the sample.

The above correction method was also applied to determine Hg in bovine kidneys. The ^{203}Hg and ^{75}Se nuclides emit gamma-rays of 279.2 and 279.5-keV, respectively. These two peaks could not be resolved by a detector and the half-lives are relatively long making discrimination by decay time impossible. However, ^{75}Se also emits an interference-free gamma-ray at 265 keV. The relative intensities of the interference-free and interfered peaks for ^{75}Se were measured in a Se standard. This value was then used to calculate the contribution of ^{75}Se in 279-keV peak.

The ^{56}Mn nuclide can be measured using two photopeaks at the 846 and 1811 keV. The higher energy peak is not sensitive enough to be used for detecting low concentration of Mn in a sample. The lower energy peak which is quite sensitive suffers spectral interference from the 843-keV peak of ^{27}Mg. Because the half-life of ^{27}Mg (9.45 min) is about 15-times shorter than that of ^{56}Mn (2.58 h), samples were counted to determine Mg through the 1014-keV peak of ^{27}Mg after a short decay (t_d = 1 min); then allowed to decay for 1 h and counted again for Mn using the 846-keV peak of ^{56}Mn. By that time, only about 0.5% of initial ^{27}Mg activity was present in the sample and the 846-keV peak could be reliably integrated.

2.3.8 Detection Limit

In a complex sample, high sensitivity of a trace element is not enough to give a satisfactory determination. The lowest level of an element that can be measured is limited by changes in the blank signal. However, in NAA the contribution from counting statistics is generally the most important source of variability near the detection limit. Currie [65] presented an excellent review of the statistical problems associated with qualitative and quantitative determinations in radiochemistry. According to Currie, there are three specific quality levels for a measurement:

(i) the decision limit (L_C) which is defined as the net instrument response above which an observed signal can be reliably recognized;

(ii) the detection limit (L_D) which is defined as the true net signal that may be expected *a priori* to lead to detection; and

(iii) the determination limit (L_Q) which is defined as a level of measurement with adequate precision for a quantitative determination.

All three levels can be calculated using the following formulae given by Currie [65]:

$$L_C = 2.33\sqrt{\mu_B} \tag{2.16}$$

$$L_D = 2.71 + 4.65\sqrt{\mu_B} \tag{2.17}$$

$$L_Q = 50(1 + \sqrt{1 + \frac{\mu_B}{12.5}}) \qquad (2.18)$$

where μ_B corresponds to the number of counts in the background under the peak of interest. All three levels are expressed in counts.

In the present work, detection limit based on the equation (2.17) was calculated for the elements present in whole bovine kidney sample and in the homogenate. This (L_D) is the most commonly used detection limit in nuclear analytical chemistry.

2.4 RESULTS AND DISCUSSION

2.4.1 Quality Assurance

Assurance of quality (accuracy and precision) in trace element analysis includes establishing the effectiveness of the analytical process first and then maintaining continuous quality control and quality assessment of the obtained results. The quality assessment is divided further into (i) internal quality assessment, and (ii) external quality assessment, depending on whether any other source of assistance, except the primary laboratory, is needed to make the assessment. To provide a high level of confidence in the results, several steps were followed. They are discussed in detail in the following sections.

2.4.1.1 Internal Quality Assessment

2.4.1.1.1 Neutron Flux

The measurement of composition, spatial distribution, homogeneity, stability and reproducibility of neutron flux of the Dalhousie SLOWPOKE-2 reactor has been reported by Ryan *et al.* [66], [67]. The reactor flux consists of a mixture of thermal, epithermal and fast neutrons. The thermal neutron flux was measured using the ^{60}Co activity produced by the $^{59}Co(n,\gamma)^{60}Co$ reaction in cobalt flux wires. The epithermal neutron flux was measured using the ^{198}Au activity produced by the $^{197}Au(n,\gamma)^{198}Au$ reaction and using the ^{60}Co activity produced by the $^{59}Co(n,\gamma)^{60}Co$ reaction in gold and cobalt flux wires, respectively, wrapped in 1.00 mm-thick cadmium foil. The fast neutron flux was determined on the basis of the following reactions: $^{32}S(n,p)^{32}P$, $^{54}Fe(n,p)^{54}Mn$ and $^{19}F(n,2n)^{18}F$.

The inner sites had low variations in vertical and radial flux homogeneity, *i.e.* < 0.5% cm^{-1}. The flux in the outer sites also had < 0.5% cm^{-1} variation in vertical homogeneity, but a higher variation (*ca.* 6% cm^{-1}) in the radial homogeneity [66]. Consequently, the positioning of smaller sample vials inside the large vials used for irradiations in the outer sites must be done reproducibly. The variation in stability and reproducibility of the reactor flux was less than 2% [67].

2.4.1.1.2 Calibration of Gamma-Ray Spectrum

The gamma-ray energy spectrum was frequently calibrated using the following isotopic standards: (i) ^{60}Co (gamma-ray of 1332-keV); (ii) ^{137}Cs (gamma-

ray of 661.6-keV); and (iii) ^{22}Na (gamma-rays of 511-keV and 1274.6-keV).

2.4.1.1.3 Selection of Target Isotopes and Nuclides

Target isotopes have to be carefully selected with respect to their isotopic abundance and cross section, and competing reactions that may occur during activation. Also selecting a proper nuclide (and gamma-ray) with respect to convenient half-life, gamma-ray energy and no or least interferences are of great importance.

Selenium was determined in this work *via* its short lived nuclide ^{77m}Se. The 162-keV photopeak of ^{77m}Se may suffer spectral interference from the 162.4-keV photopeak of $^{116m2}In$ (half-life of 2.18 s). However, this interference was negligible due to a decay time of 10 s. Over that time, the activity of $^{116m2}In$ decreased by almost 5-fold with only about half-fold decrease of the ^{77m}Se activity. For comparison purposes, the Se content was also determined *via* its long-lived nuclide ^{75}Se (gamma-ray of 265 keV). Data for NIST Rice Flour SRM 1568 and NIST Bovine Liver SRM 1577a are presented in Tables 2.2 and 2.3, respectively. It can be seen that the agreement of results using the two nuclides was very good. However, ^{75}Se was not routinely used in this study due to its long half-life (120.4 d) which makes the analysis of a large number of samples very time consuming.

In this work, the Br content was determined via ^{82}Br (half-life of 1.47 d).

Table 2.2

Selenium in NIST Rice Flour SRM 1568

Nuclide	This Work[a] (ppm)	Certified Value (ppm)
^{77m}Se	0.34 ± 0.01	0.40 ± 0.10
^{75}Se	0.33 ± 0.01	0.40 ± 0.10

[a] average of 7 determinations.

Table 2.3

Selenium in NIST Bovine Liver SRM 1577a

Nuclide	This Work[a] (ppm)	Certified Value (ppm)
^{77m}Se	0.71 ± 0.02	0.71 ± 0.01
^{75}Se	0.72 ± 0.03	0.71 ± 0.01

[a] average of 7 determinations.

Attempts were made to use ^{80}Br which has a shorter half-life (17 min), but the results obtained were less accurate, most likely due to the spectral interference from a double escape peak of ^{38}Cl at 620 keV to the 617-keV peak of ^{80}Br.

Zinc concentrations were measured using ^{69m}Zn nuclide (half-life of 13.8 h). Zinc can be also determined *via* its long-lived nuclide ^{65}Zn (half-life of 243.8 d). Data obtained for NIST Wheat Flour SRM 1567 and NIST Bovine Liver SRM 1577a using both nuclides are presented in Tables 2.4 and 2.5, respectively. It can be seen that the results are of the same accuracy and precision using either nuclide. The use of the nuclide of shorter half-life, however, allowed us to significantly shorten the time of analysis.

2.4.1.1.4 Corrections for Interferences

Aluminum concentrations, corrected for P, were determined in four RMs and SRMs, namely Rice Flour (NIST SRM 1568), Bovine Liver (NIST SRM 1577a), Dogfish Liver (NRC RM DOLT-1), and Dogfish Muscle (NRC RM DORM-1). Since cadmium ratios were 1.15 for P and 18.2 for Al, the simplified formula (2.11) was used in all calculations. The results obtained are shown in Table 2.6. It can be seen that the contribution of P to Al is significant, ranging from 15% in Dogfish Muscle to 75.8% in Bovine Liver. Only in case of Bovine Liver was there a reported value of 2 ppm Al which compares well with our data.

The P contribution to Al concentration was calculated for a kidney and its subcellular fractions and data are presented in Table 2.7. It is evident that the

Table 2.4

Zinc in NIST Wheat Flour SRM 1567

Nuclide	This Work[a] (ppm)	Certified Value (ppm)
^{69m}Zn	11.1 ± 0.5	10.6 ± 1.0
^{65}Zn	11.7 ± 0.6	10.6 ± 1.0

[a] average of 7 determinations.

Table 2.5

Zinc in NIST Bovine Liver SRM 1577a

Nuclide	This Work[a] (ppm)	Certified Value (ppm)
^{69m}Zn	137 ± 12	123 ± 8
^{65}Zn	142 ± 11	123 ± 8

[a] average of 7 determinations.

Table 2.6

Phosphorus corrections for determining aluminum in reference materials

Material Analyzed	Al (ppm)			P Contribution to Al (%)
	Not Corrected for P	Corrected for P	Reported Value	
Rice Flour NIST SRM 1568	3.00 ± 0.10	2.10 ± 0.17	N/A	30.0
Bovine Liver NIST SRM 1577a	7.07 ± 0.20	1.71 ± 0.36	2.0	75.8
Dogfish Liver NRC RM DOLT-1	12.71 ± 0.94	8.18 ± 0.54	N/A	35.6
Dogfish Muscle NRC RM DORM -1	33.42 ± 0.91	28.4 ± 2.6	N/A	15.0

N/A = value not available.

Table 2.7

Phosphorus corrections for determining aluminum in bovine kidney and its subcellular fractions[a]

Sample Analyzed	Al (ppm)		P Contribution to Al (%)
	Not Corrected for P	Corrected for P	
Whole Kidney	7.50 ± 0.54	3.45 ± 0.36	54.0
Homogenate	3.66 ± 0.20	1.90 ± 0.09	48.1
Nuclei	5.25 ± 0.04	2.53 ± 0.02	51.8
Mitochondria	5.56 ± 0.79	3.07 ± 0.15	44.8
Lysosomes	7.59 ± 0.84	3.81 ± 0.81	49.8
Microsomes	13.51 ± 1.47	6.57 ± 0.32	45.9
Cytosol	1.88 ± 0.09	1.01 ± 0.04	46.6

[a] results expressed as μg of element per g of dry mass.

uncorrected values are twice as high as the corrected values. This is due to the fact that the concentration of P in biological samples is, on the average, three orders of magnitude higher than the concentration of Al [68]. Phosphorus interferences in Al activity is a common problem in determining Al in biological samples and has been discussed by several researchers, *e.g.*, Al-Hashimi *et al.* [69].

Corrections in ^{27}Mg activity for Al interference were made in the same four reference materials analyzed previously for Al and P. Cadmium ratios were 1.2 for Al and 22.3 for Mg, therefore the simplified formula (2.11) was used in all corrections. The results presented in Table 2.8 indicate that Al contribution to the measured Mg concentration is negligible, in the range of 1.5 - 2.5% in all samples analyzed. Interference is small due to the fact that the concentration of Al in biological samples is about three orders of magnitude lower than the concentration of Mg [68]. The above correction was applied to three different kidney samples and the results are presented in Table 2.9. Again, only 1.3 - 2.6% of total ^{27}Mg activity originated from Al. Based on the above results, Mg determinations in all other samples were done without correcting for the interference from Al.

To determine F concentrations in samples analyzed throughout this work, the ratio of two ^{24}Na peaks as described in Section 2.3.7 was calculated. This ratio was measured as 11.6 in the half-full small vial geometry and 16.4 in the medium-size vial geometry.

In order to evaluate Hg levels in the samples, the ratios of two ^{75}Se

Table 2.8

Aluminum corrections for determining magnesium in reference materials

Material Analyzed	Mg (ppm)			Al Contribution to Mg (%)
	Not Corrected for Al	Corrected for Al	Certified Value	
Rice Flour NIST SRM 1568	495 ± 30	485 ± 27	N/A	2.0
Bovine Liver NIST SRM 1577a	641 ± 76	625 ± 56	600 ± 15	2.5
Dogfish Liver NRC RM DOLT-1	992 ± 50	976 ± 54	1100 ± 150	1.6
Dogfish Muscle NRC RM DORM -1	1087 ± 52	1070 ± 70	1210 ± 130	1.5

N/A value not available.

Table 2.9

Aluminum corrections for determining magnesium in bovine kidneys[a]

Sample	Mg (ppm)		Al Contribution to Mg (%)
	Not Corrected for Al	Corrected for Al	
Kidney 1	194.6 ± 7.6	191.9 ± 7.3	1.4
Kidney 2	132.4 ± 4.8	129.0 ± 1.6	2.6
Kidney 3	164.3 ± 9.9	162.1 ± 6.4	1.3

[a] results expressed as µg per g of fresh sample.

photopeaks as described in Section 2.3.7 were determined. The ratio was 2.4 for both geometries.

2.4.1.1.5 Optimization of Sample Geometry

The sample geometry was optimized for the determination of Se, the element of primary interest in this study. Two sample geometries were selected, namely quarter and half volumes of the small-size irradiation vials. The NIST Rice Flour SRM 1568 and NIST Bovine Liver SRM 1557a were analyzed for this purpose and the results are presented in Tables 2.10 and 2.11, respectively. As expected, the half-full-vial geometries had larger masses and gave better accuracy in both cases. This sample geometry was therefore selected for use in further studies.

2.4.1.1.6 Correction for Blanks

The accuracy of measurement at low levels of trace elements can be highly affected if one does not consider all sources of sample contamination. There are four basic origins of contamination which produce an analytical blank: (i) the environment where the analysis is performed; (ii) the reagents used in experiments; (iii) the researcher performing the analysis; and (iv) the instrument itself.

Since no "clean room" (such as the class 100 type) was available, all experiments in this work were performed in an environment as clean as possible,

Table 2.10

Optimization of sample geometry for the determination of selenium in NIST Rice Flour SRM 1568

Sample Geometry	Sample Mass (g)	This Work[a] (ppm)	Variation from Certified Value (%)
Quarter-full Vial	0.1921 ± 0.0075	0.29 ± 0.01	27.5
Half-full Vial	0.3756 ± 0.0113	0.34 ± 0.01	15.0

[a] average of 7 determinations;

Certified value: 0.40 ± 0.10 ppm.

Table 2.11

Optimization of sample geometry for the determination of selenium in NIST Bovine Liver SRM 1577a

Sample Geometry	Sample Mass (g)	This Work[a] (ppm)	Variation from Certified Value (%)
Quarter-full Vial	0.2054 ± 0.0082	0.66 ± 0.04	7.0
Half-full Vial	0.3671 ± 0.0147	0.71 ± 0.02	< 0.02

[a] average of 7 determinations;

Certified value: 0.71 ± 0.02 ppm.

for example, in well-ventilated rooms and on frequently cleaned benches. Reagent blanks were determined by analyzing all chemicals used for the presence of impurities; the data are presented in Chapters 3, 4 and 5. Wherever possible, chemicals with highest purity were employed. The water used was first distilled in a quartz apparatus and then deionized using an ultrapure deionization column from the Fisher Scientific. The quality of the water in terms of trace element contaminants was periodically checked in our laboratory [70] and the water is considered to be of high purity. A clean labcoat was worn by the analyst while the analyses were performed and hands were often washed in order to diminish a blank arising from physical contact of the analyst with the sample. Glasswares were washed in order to remove as many contaminants as possible; the washing procedure is described in the next Chapter, Section 3.2.1.

Analysis by neutron activation always ended a long chain of biochemical and separation experiments and all possible sources of analytical blank in this step were also recognized. Polyethylene vials, either empty or containing only a sucrose matrix, were subjected to the same handling, irradiation, decay and counting scheme as were samples or comparator standards. It enabled the concentration of any element under study to be corrected for reagent blanks. Nuclides present in the vials and in the sucrose are shown in Table 2.12. Eight elements, namely Al, Au, Br, Cl, K, Mg, Mn and V were detected in vials and their concentrations were taken into consideration in calculating levels of these elements in all samples analyzed. The levels of Mg and V were found to be

Table 2.12

Elements present in polyethylene irradiation vials and sucrose[a]

Element	Concentration (ppm)	
	Polyethylene Vial	Sucrose
Al	0.33 ± 0.04	< 0.05
Au	0.017 ± 0.003	< 0.008
Br	0.022 ± 0.003	0.084 ± 0.008
Cl	2.17 ± 0.33	2.76 ± 0.37
K	0.92 ± 0.13	4.54 ± 0.22
Mg	20.18 ± 3.43	< 1.65
Mn	0.007 ± 0.001	< 0.005
Na	< 0.003	0.96 ± 0.05
V	0.94 ± 0.09	< 0.002

[a] average of 8 determinations.

especially high and comparable to the levels of these elements in biological and other samples analyzed. Neglecting these values in the calculations would have introduced a very large systematic error. A high relative standard deviation observed (±10% or greater) indicated great variability among vials.

Sucrose was found to be contaminated with Br, Cl, K and Na. Appropriate corrections were made to elemental standards.

2.4.1.1.7 Analyses of Replicate Standards (Control Charts)

The classical means to evaluate the precision of a method is to perform measurement of replicate samples. For this purpose, comparator standards were tested in replicates of five to seven and the results are presented here as a mean ± 1 standard deviation (SD).

Constructing quality control charts is also a basic practice in assessing the quality of the results produced. In this project, the control chart format developed by Shewhart [71] was used. A central line was defined as an average of all measurements, upper and lower warning limits as ± 2 SD and control limits as ± 3 SD. In addition, limits of ± 1 SD were included in charts as in the present work the results are given as a mean ± 1 SD. One nuclide from each type of irradiation, decay and counting scheme was selected for presentation here. Results of the determination of Se *via* ^{77m}Se, Se *via* ^{75}Se, Cu *via* ^{66}Cu and Zn *via* ^{69m}Zn are presented in Figs. 2.1 through 2.4. Solutions of the following concentrations were used: Se (^{77m}Se): 50 µg mL^{-1}; Se (^{75}Se): 1000 µg mL^{-1}; Cu: 500 µg mL^{-1}; and Zn:

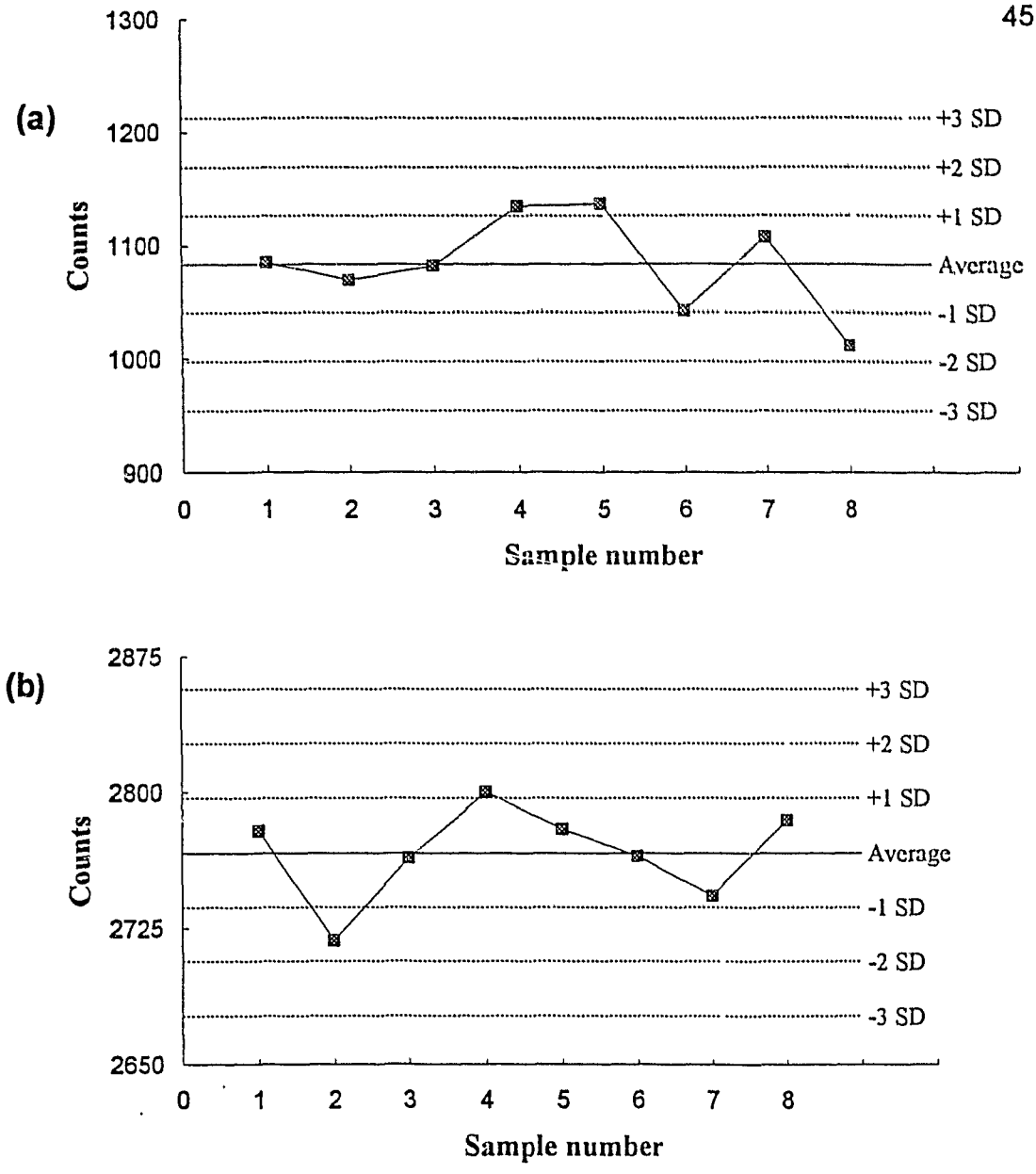


Fig. 2.1. Quality control charts for Se determined *via* ^{77m}Se.

(a) small vial geometry; t_i = 30s; t_d = 10s; t_c = 30s;

(b) medium vial geometry; t_i = 1 min; t_d = 10s; t_c = 1min.

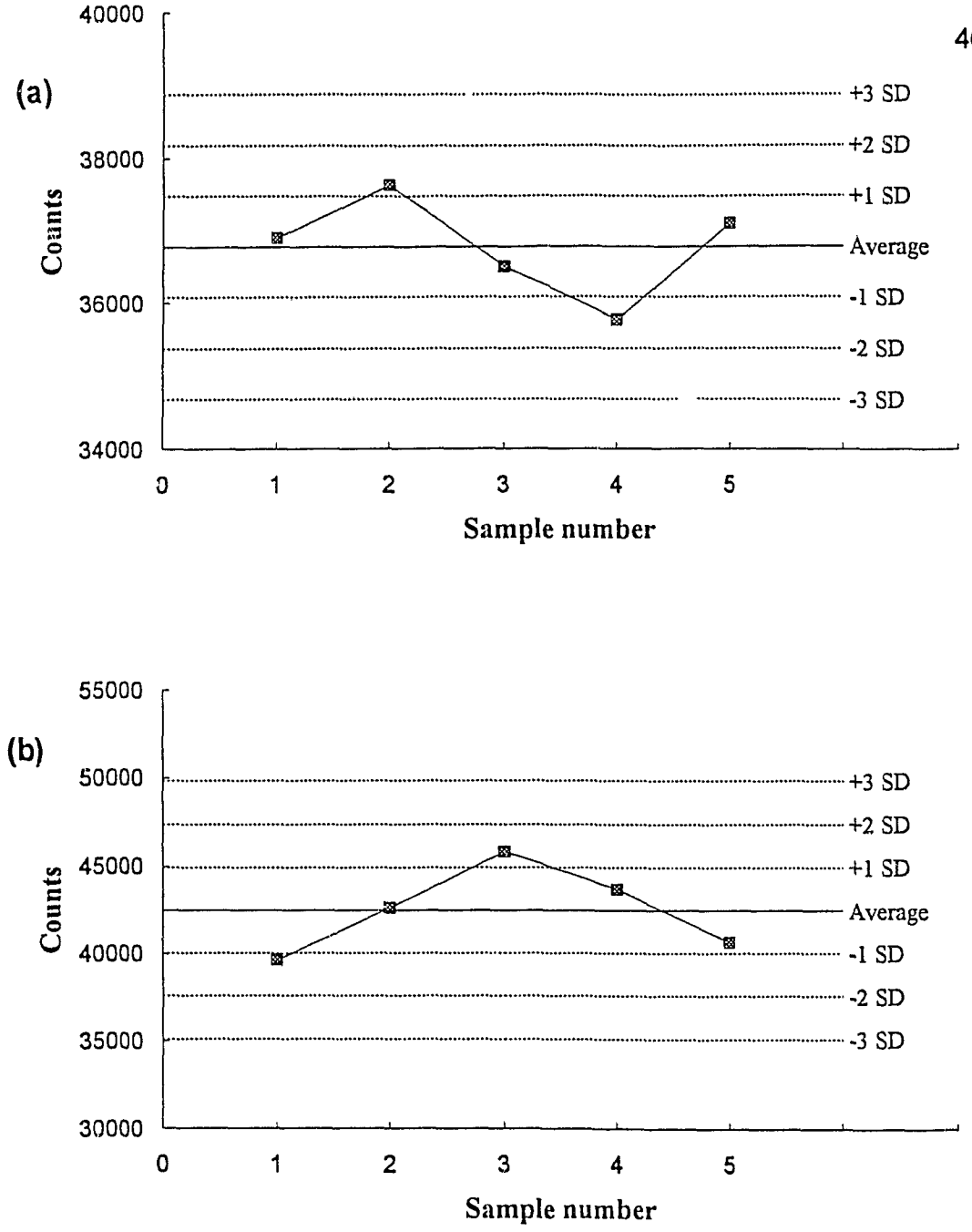


Fig. 2.2. Quality control charts for Se determined *via* ^{75}Se.

(a) small vial geometry; t_i = 7 h; t_d = 16 d; t_c = 1 h;

(b) medium vial geometry; t_i = 7 h; t_d = 16 d; t_c = 1 h.

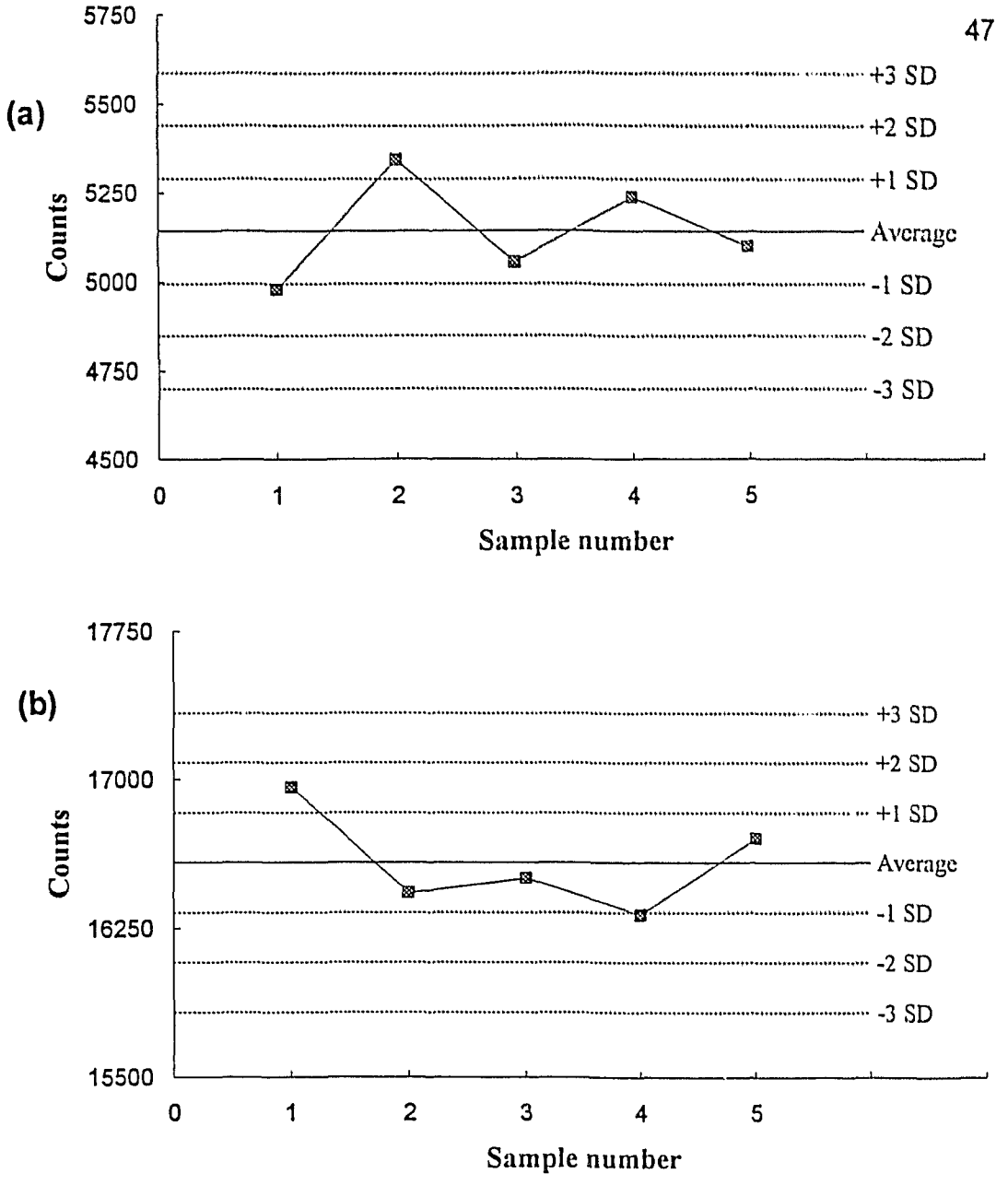


Fig. 2.3. Quality control charts for Cu determined *via* ^{66}Cu.

(a) small vial geometry; t_i = 5 min; t_d = 1min; t_c = 10 min;

(b) medium vial geometry; t_i = 5 min; t_d = 1min; t_c = 10 min.

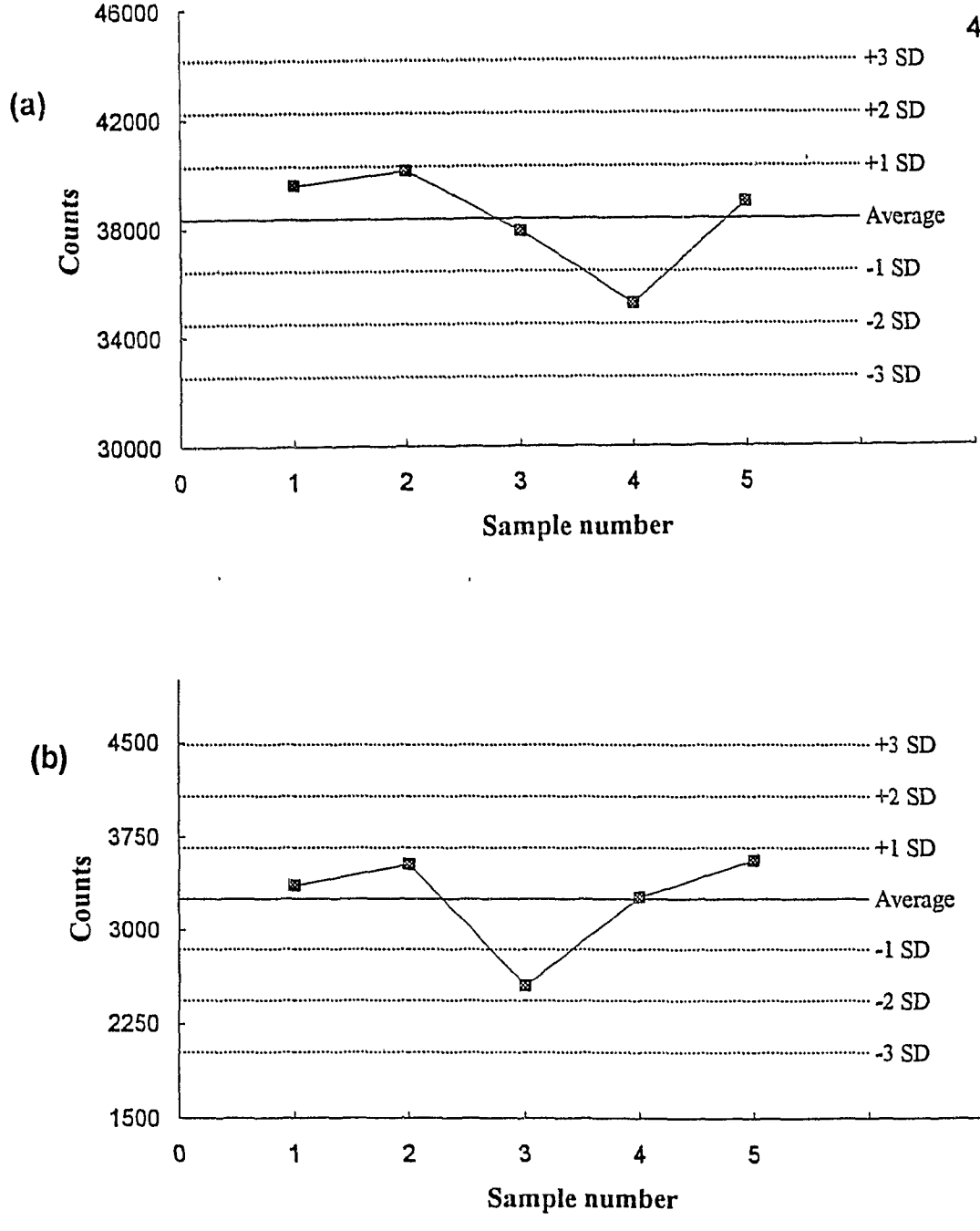


Fig. 2.4. Quality control charts for Zn determined *via* ^{69m}Zn.

(a) small vial geometry; t_i = 7 h; t_d = 16 h; t_c = 0.5 h;

(b) medium vial geometry; t_i = 7 h; t_d = 16 h; t_c = 0.5 h.

100 μg mL^{-1}. Each comparator standard was studied in both sample geometries. Standards were analyzed in order of sample number and the variation could be due to counting statistics and sampling errors. As can be seen, all deviations among the results remained well within the upper and lower warning limits and no systematic trend was observed with respect to time. The sample geometry did not affect the overall conclusions.

2.4.1.2 External Quality Assessment

Accuracy and systematic errors are traditionally linked together. Such errors are detected when the same samples are analyzed by other laboratories or other analytical techniques. This might be done by a simple exchange of standards or reference materials, *i.e.* substances of known compositions. A reference material should be indistinguishable from analyzed sample materials with respect to: (i) overall level of trace element concentrations; (ii) chemical and physical form; and (iii) matrix composition.

In this work, the accuracy of the INAA methods developed was evaluated by analyzing five reference and standard reference materials. The results are presented in Tables 2.13 through 2.17 for the analyses using half-full small vial geometry and in Table 2.18 for the Bovine Liver SRM analyzed using a geometry of the medium-size irradiation vial.

A t-test was performed to compare the resulting means with their certified or reported values to see if the results were statistically distinguishable at the 95%

Table 2.13

Elemental content of NIST Wheat Flour SRM 1567 by INAA

Element	This Work (ppm)[a]	Certified Value (ppm)
Br	6.1 ± 0.05	(9.0)
Ca	209 ± 20	190 ± 10
Cl	588 ± 18	N/A
K	1410 ± 68	1360 ± 39
Mg[b]	374 ± 15	N/A
Mn	9.1 ± 0.4	8.5 ± 0.5
Mo	1.50 ± 0.24	(1.6)
Na	8.6 ± 0.3	8.0 ± 1.5
Se[c]	1.18 ± 0.04	1.1 ± 0.2
Zn	11.1 ± 0.5	10.6 ± 1.0

(Information value);

N/A = not available;

[a] average of 5 measurements;

[b] corrected for Al interference;

[c] determined using ^{77m}Se.

Table 2.14

Elemental content of NIST Rice Flour SRM 1568 by INAA

Element	This Work (ppm)[a]	Certified Value (ppm)
As	0.34 ± 0.02	0.41 ± 0.05
Br	0.95 ± 0.06	(1.0)
Cl	250 ± 6	N/A
K	1010 ± 40	1120 ± 20
Mg[b]	495 ± 30	N/A
Mn	20.1 ± 0.4	20.1 ± 0.4
Mo	1.50 ± 0.24	(1.6)
Na	5.78 ± 0.32	6.0 ± 1.5
Rb	8.89 ± 1.26	(7.0)
Se[c]	0.34 ± 0.01	0.40 ± 0.10
Zn	17.1 ± 0.9	N/A

(Information value);

N/A = not available;

[a] average of 7 measurements;

[b] corrected for Al interference;

[c] determined using ^{77m}Se.

Table 2.15

Elemental content of NIST Bovine Liver SRM 1577a by INAA

Element	This Work (ppm)[a]	Certified Value (ppm)
Al[b]	1.71 ± 0.36	(2)
Br	9.05 ± 0.17	(9)
Cl	3030 ± 10	2800 ± 980
Cu	166 ± 4	158 ± 7
Fe	205 ± 9	194 ± 20
K	9300 ± 102	9960 ± 70
Mg[c]	641 ± 76	600 ± 15
Mn	10.5 ± 0.3	9.9 ± 0.8
Mo	3.7 ± 0.1	3.5 ± 0.5
Na	2310 ± 30	2430 ± 130
Rb	13.6 ± 1.5	12.5 ± 0.1
Se[d]	0.72 ± 0.05	0.71 ± 0.01
Zn	137 ± 12	123 ± 8

(Information value);

[a] average of 6 measurements;

[b] corrected for P interference;

[c] corrected for Al interference;

[d] determined using ^{77m}Se.

Table 2.16

Elemental content of NRC Dogfish Muscle RM DORM-1 by INAA

Element	This work (ppm)[a]	Certified Value (ppm)
Al[b]	28.4 ± 2.6	N/A
As	17.1 ± 0.3	17.7 ± 2.1
Br	46.1 ± 0.5	N/A
Cl	1.17 ± 0.02	1.13 ± 0.03
Cr	4.07 ± 0.55	3.60 ± 0.40
Fe	68.4 ± 4.0	63.6 ± 3.3
K, %	1.54 ± 0.03	1.59 ± 0.1
Mg[c]	1090 ± 50	1210 ± 130
Mn	1.78 ± 0.40	1.32 ± 0.26
Se[d]	1.54 ± 0.08	1.62 ± 0.12
Na	7600 ± 200	8000 ± 600
Zn	19.4 ± 3.6	21.3 ± 1.0

N/A = not available;

[a] average of 7 measurements;

[b] corrected for P interference;

[c] corrected for Al interference;

[d] determined using ^{77m}Se.

Table 2.17

Elemental content of NRC Dogfish Liver RM DOLT-1 by INAA

Element	This Work (ppm)[a]	Certified Value (ppm)
Al[b]	8.18 ± 0.54	N/A
As	10.4 ± 0.6	10.1 ± 1.4
Br	20.5 ± 1.4	N/A
Cl	6690 ± 180	6880 ± 220
Fe	748 ± 43	712 ± 48
K	9420 ± 1000	10100 ± 1000
Mg[c]	992 ± 50	1100 ± 150
Mn	9.36 ± 0.80	8.72 ± 0.53
Na	7400 ± 80	7260 ± 730
Se[d]	7.03 ± 0.29	7.34 ± 0.42
Zn	86.7 ± 1.8	92.5 ± 2.3

N/A = not available;

[a] average of 7 measurements;

[b] corrected for P interference

[c] corrected for Al interference;

[d] determined using ^{77m}Se.

Table 2.18

Elemental content of NIST Bovine Liver 1577a SRM by INAA

Element	This Work (ppm)[a]	Certified value (ppm)
Ag	0.09 ± 0.03	0.04 ± 0.01
Br	9.0 ± 0.4	(9)
Cl	2570 ± 18	2800 ± 980
Co	0.20[b]	0.21 ± 0.05
Cu	149 ± 2	158 ± 7
Fe	199 ± 5	194 ± 20
K	9570 ± 140	9960 ± 70
Mg[c]	562 ± 82	600 ± 15
Mn	8.9 ± 0.4	9.9 ± 0.8
Mo	3.8 ± 1.3	3.5 ± 0.5
Na	2260 ± 50	2430 ± 130
Rb	12.1 ± 1.3	12.5 ± 0.1
Se[d]	0.71 ± 0.02	0.71 ± 0.01
Zn	137 ± 14	123 ± 8

(Information value);

[a] average of 7 measurements;

[b] corrected for Al interference;

[c] one sample analyzed;

[d] determined using ^{77m}Se.

confidence level. No statistical difference was found in 57% of all elements determined. Additionally, the difference between the experimental value and the certified value, relative to the certified value, was calculated for each element. For 82% of the elements these differences were within ±10% of the certified values. The values for Se in all materials analyzed were statistically indistinguishable from the certified values.

The agreements for some elements such as K, Mg, and Na were relatively poor in all analyzed materials. The concentration of Ag detected in Bovine Liver samples (Table 2.18) was twice as high as the certified value. This discrepancy was due to the very low level of Ag in the sample, approaching the detection limit of 0.07 ppm of the INAA method used. Cobalt in Bovine Liver SRM was determined on the basis of only one sample. In other samples, Co could not be accurately measured as the amount was close to its detection limit of 0.15 ppm.

Neutron activation analysis is recognized as a technique of high inherent accuracy and this feature makes it an excellent method when maximum accuracy is required such as in the analysis of reference materials. Greenberg [72], for example, has examined the extensive use of NAA to certify SRMs at the U.S. National Institute of Standards and Technology (NIST).

In conclusion, the accuracy of the INAA method developed here was found to be good for the majority of the samples analyzed.

2.4.2 Detection Limits

The detection limits, L_D, according to Currie [65] for several elements were calculated in a sample of an intact bovine kidney and in a sample of homogenate of the kidney. Results are presented in Table 2.19. The nuclides and gamma-rays used are listed in Table 2.1. Timing parameters used are described in Section 2.4.3.

The preparation of the homogenate is described in Section 3.3.2 of Chapter 3. Before homogenization, tissue was disrupted in a blender and washed once with a buffer. Since upon blending cells were not broken, it was assumed that the wash (meant to remove some of the residual blood) would not remove elements present in cell fractions as attached to proteins. This process of washing was also supposed to remove most of Na and Cl which otherwise would contribute highly to the background activity and increase the dead time. Researchers using NAA to study elemental content of biological materials know that ^{24}Na and ^{38}Cl are the dominant activities in freshly irradiated tissue samples [73]. Any method that removes these unwanted activities is therefore highly sought. As can be seen from Table 2.19, simple washing improved detection limits by about two-fold for Ag, Al, Br, Ca, Cd, Co, Cr, F, Fe, Hg, Mo, Rb, Sb, Sc, Se, Sn, W and Zn; by four-fold for As and K; by 5-fold for Cu and S; by 7-fold for I, Mg and Mn; by 8-fold for Cl and V; and by 9-fold for Na. The detection limits for the majority of the elements were on a ppb level.

Table 2.19

Detection limits of elements present in bovine kidney and homogenate by INAA

Element	Detection Limit (L_D)[a]	
	Whole Kidney	Homogenate[b]
Al	606 ppb	270 ppb
Ag	52.6 ppb	21.9 ppb
As	43.0 ppb	11.1 ppb
Br	105 ppb	51.1 ppb
Ca	81.5 ppm	48.4 ppm
Cd	525 ppb	281 ppb
Cl	32 ppm	4.15 ppm
Co	79.2 ppb	53.6 ppb
Cr	602 ppb	268 ppb
Cu	2.52 ppm	524 ppb
F	46.8 ppm	16.7 ppm
Fe	41.8 ppm	18.1 ppm
Hg	290 ppb	167 ppb
I	211 ppb	31.7 ppb
K	35.4 ppm	9.61 ppm
Mg	128.4 ppm	18.1 ppm
Mn	209 ppb	30.4 ppb
Mo	234 ppb	108 ppb
Na	31.3 ppm	3.45 ppm
Rb	2.37 ppm	887 ppb
S	0.343 %	664 ppm
Sb	32.8 ppb	17.8 ppb
Sc	6.4 ppb	3.9 ppb
Se	41.1 ppb	16.1 ppb
Sn	18.3 ppm	10.0 ppm
V	62.0 ppb	7.4 ppb
W	86 ppb	36 ppb
Zn	3.57 ppm	2.24 ppm

[a] calculated using the equation (2.17): $L_D = 2.71 + 4.65\sqrt{\mu_B}$

[b] after washing.

2.5 SUMMARY

Timing parameters for INAA were selected so that multielement analysis of samples could be performed. The following four irradiation, decay, and counting schemes were applied: (i) t_i = 1 min, t_d = 10 s, t_c = 1 min to detect short-lived nuclides of Ag, F, Rb and Se; (ii) t_i = 5 min, t_d = 1 min, t_c = 10 min to detect short- and medium-lived nuclides of Al, Ca, Cl, Cu, Mg, Mn, Na, S and V; (iii) t_i = 7 h, t_d = 16 h, t_c = 0.5 h to detect medium-lived nuclides of As, Br, Cd, K, Mo, W and Zn; and (iv) t_i = 7 h, t_d = 16 d, t_c = 1 h to detect long-lived nuclides of Co, Cr, Fe, Hg, Sc, Se and Sn. In order to measure short-lived nuclides in materials with high Na and Cl contents, samples had to be irradiated for a shorter length of time (t_i = 1 min) in order to prevent a significant build up of background activity and a high dead time.

The quality assurance (QA) program involved various steps including replicate analysis of internal standards and certified reference materials, the analysis of chemicals used for the presence of impurities, selection of proper nuclides, *etc.* The precision of the INAA method was found to be well within ± 2 SD. The accuracy of the method determined by analyzing several certified reference materials was found to be good: 57% of all experimental results were statistically indistinguishable from the certified values at 95% confidence interval, and 82% of our values were within ± 10% of the certified values.

Several spectral and competing reaction interferences were recognized. These were: (i) interference of P in the Al determination; (ii) interference of Al in

the Mg determination; (iii) interference of Na in the F determination; and (iv) interference of Se in the Hg determination. Appropriate corrections were made in all cases except for the Mg determination, where the contribution of Al was found to be < 3%.

The detection limits of the elements observed in bovine kidney and its homogenate were calculated according to Currie's method [65]. The values obtained for kidney samples were quite high due to the high Na and Cl contents of the samples analyzed. A single washing of disrupted kidney tissue in the homogenate preparation removed large amounts of Na and Cl and allowed for a significant decrease in the detection limits of the elements investigated.

3. DEVELOPMENT OF BIOANALYTICAL METHODS

3.1 INTRODUCTION

The measurement of total elemental content is always useful. It is the first step in any further fractionation and speciation studies. The determination itself is a laborious task, and in many circumstances the different sources of errors have only recently been recognized. Certain studies, however, require more specific information regarding the presence of elemental species. This is because the metabolic behavior and the essentiality or toxicity of an element largely depend on its chemical forms.

In the first part of this chapter, a brief introduction is given on the various methods of trace element-protein speciation. Next, biochemical and instrumental analytical techniques applied to study protein-bound elements are discussed. The following part describes the bioanalytical methods developed to study trace elements in bovine kidney and its subcellular fractions. This chapter presents more of the initial techniques that needed to be applied prior to protein separation and purification steps which are described in Chapters 4 and 5.

3.1.1 Trace Element Speciation

Trace elements exist in living materials in several chemical forms. In general, these different species can be divided into two groups: (i) compounds of relatively low molecular weight; and (ii) macromolecules. The application of an

appropriate speciation method depends on the type of species to be studied. Majority of the elemental species that are potentially harmful to living organisms exist as small molecules. Many metals are capable of forming low molecular weight organometallic compounds which are very toxic. Examples include arsenic trichloride and antimony trichloride [74], cadmium chloride [75], [74], tetraethyllead [76], dimethylmercury [77], [74], dimethylselenium [78].

The total content of an element does not necessarily relate directly to the amount of toxic species. A good example is the amount of As in urine (the major route of As excretion) of occupationally exposed persons [79]. Ingestion of sea food which is rich in As increases the level of this element in urine. However, this As is relatively low in toxicity as it is tightly bound as arsenobetaine. The excretion of inorganic As compounds such as dimethylarsenic acid or monomethylarsenic acid is not influenced by seafood intake. An indication of the As exposure can only be provided by monitoring the levels of these species.

The vital role of a trace element is quite often related to the function of that element as an active component of an enzyme or other protein, meaning a large molecule [80]. Therefore, while the speciation of small molecules is preferred in the investigations of toxicity, the speciation of large molecules is of interest in the studies of the essential biological functions of trace elements.

3.1.2 Determination of Protein-Bound Trace Elements

As already mentioned, the majority of essential functions of trace elements in living organisms stem from the association of these elements with proteins. Elements such as Fe, Cu, Cd or Zn have been known for many years as being bound to macromolecules. The total content of an element in a tissue cannot therefore be taken as a measure of the levels of biologically active metalloproteins.

If a protein in question is a metal-containing enzyme of known biological function, its determination is simplified to the determination of the enzymatic activity. Unfortunately, this does not apply to new compounds. Another possible speciation method could be immunoassay. In this method, a labelled complex of a specific antibody and the antigen (*i.e.* the species investigated) is formed, and then quantitative measurement of this complex is done by means of the label. However, for this technique to be applicable, sufficient amounts of the elemental species have to be isolated in a highly purified form for both the production of the antibody and the labelled antigen. To date, this can only be achieved with a few compounds as all that is known of many binding forms of trace elements is that they contain the element in question. Until better methods are available, fractionation and trace element analytical techniques have to be combined and speciation studies carried out by determining the amount of the element in the separated fractions.

To quantitatively study the subcellular distribution of metals in organisms, it is necessary to first isolate the different metal-binding molecules and then to

quantify various metals associated with these macromolecules. In recent years, different forms of liquid chromatography are found to be successful in separating different cytosolic and other molecules with good resolution, speed and efficiency.

Cadmium speciation by means of size exclusion HPLC coupled to ICP-MS was carried out by Crews *et al.* [81] in porcine kidney. The majority of soluble Cd was found to be bound to metallothionein-like protein (MW of 12 kDa) that was resistant to cooking and to *in vitro* gastrointestinal digest of cooked kidney. The feasibility of using HPLC-ICP-MS for the analysis of metalloproteins, particularly metallothioneins, was also investigated by Mason *et al.* [82]. Multielement detection ability, reproducibility, linearity of response, recovery of analyte and detection limits for protein-bound Cd, Cu and Zn were evaluated. It was found that the total time of analysis using coupling method was about 10 times shorter than if the fractions had been collected and analyzed individually. The detection limits were very low and varied from 240 to 350 pg of protein. It was noted, however, that protein was lost on the column and also that metals exchanged between protein and the mobile and stationary phases during chromatography.

Distribution of Cu, Fe, Mg, Pb and Zn among proteins of human and rat blood was studied by Gercken and Barnes [83] using size exclusion HPLC coupled to ICP-MS. The detection limit for Pb in protein fractions was 0.15 μg L^{-1}. The HPLC-ICP-MS system improved the sensitivity for Zn, Cu, and Pb by factors of 10, 100 and 1000, respectively. Lead was found in human serum in at least three molecular weight fractions with the highest signal being coincident with the main

ceruloplasmin. Impurities present in a buffer created problems in determination of Fe, Zn and Mg.

The possibility of a routine application of ion exchange fast protein liquid chromatography (FPLC) directly interfaced to the flame atomic absorption spectrometry (FAAS) in clinical laboratories was investigated by Ebdon *et al.* [84]. Separation of plasma proteins on the column was completed within 18 minutes. Zinc, Cu and Cd were studied in human blood plasma. The detection limits obtained were comparable with those produced by an ICP system but with simpler, more economical and better understood method of operation. Proteins associated with Zn were characterized.

Toxicity of Al is now well recognized. The total Al concentration in serum is an useful index of acute Al exposure but as the bioavailability of Al depends strongly on the type of Al compound, only speciation has any value in the assessment of Al toxicity. Aluminum in biological specimens is difficult to determine. The available methods lack sensitivity and selectivity for measuring low levels of this element in such samples. Contamination is a serious problem due to the ubiquity of Al and the use of high purity reagents and careful sample handling are absolutely necessary. Aluminum speciation in blood was performed by Keirsse *et al.* [85]. Aluminum-bound proteins in blood were separated using size exclusion chromatography and then the metal was detected by AAS. Three Al peaks eluted from the column with the overall element recovery of 90%. It was concluded that gel filtration was a valid technique for studying the speciation of Al

in biological fluids. More extensive studies were carried out by Gonzalez *et al.* [86]. Protein-binding Al in blood of patients undergoing chelation therapy was investigated using ultrafiltration and ion-exchange HPLC and metal detection was done using electrothermal atomization AAS (EAAAS). It was found that the majority of Al from healthy blood serum was protein-bound and that the main Al-containing protein was transferrin.

Neutron activation analysis has been applied for many years as an useful multielement detection technique in metal speciation. Radiochemical NAA (RNAA) was employed by Norheim and Steinnes [87] to study distribution of some selected trace elements in soluble protein fractions from normal human liver. Zinc, Cd, Hg, Cu, As, and Se were determined in each fraction after gel filtration chromatography and then metal-binding proteins characterized. Behne *et al.* [88] investigated the possibility of combining different protein separation techniques, *e.g.*, ultrafiltration, gel filtration chromatography, gel electrophoresis and isoelectric focusing with instrumental NAA (INAA). A need for precautions due to the possibility of sample contamination was stressed. Sabbioni *et al.* studied V in different body fluids and tissues in rat [89] and in human blood [90]. Radiotracer experiments were performed using ^{48}V injected intravenously in rats. The intracellular distribution of the radioactive isotope was then studied in different tissues, blood and urine fractions using gel filtration, ion exchange chromatography, gel electrophoresis and NAA techniques. In human blood, V was found to be associated with the Fe-binding protein, transferrin.

Protein-bound metal species in bovine kidneys were investigated by Jayawickreme and Chatt [91], [48], [92]. Different subcellular fractions were studied for trace element content using INAA and kidney supernatant was further characterized by gel filtration, ion exchange chromatography, chromatofocusing, electrofocusing and fractionation by ammonium sulfate precipitation. The work was focused mainly on protein-bound Cu, Mn and Zn, but as INAA permits multielemental studies, the levels of other trace elements were also measured.

As can be seen from this short survey, various methods are used to study trace elements associated with proteins. The method applied depends on the type of analyzed species and quite often on the accessibility to a particular instrument. The main problem faced by all researchers is contamination as small amounts of biological materials often come in contact with large quantities of chemicals. The apparatus and materials used in the separation processes have not yet been fully designed for these types of experiment. Unless highly pure materials and chemicals are commercially available, considerable effort has to be made to recognize and eliminate errors due to contamination. Another important issue for consideration is the type of existing reference materials for quality control purposes. To date, only a few such materials are certified for elemental species and they are not widely available.

3.2 EXPERIMENTAL

3.2.1 Chemicals and Glasswares

All chemicals and reagents were purchased from the Sigma Chemicals, USA, unless otherwise noted. All glasswares were cleaned by soaking in 4 M nitric acid for 48 h and then rinsed several times with water. Quartz-distilled deionized water was used throughout this work.

3.2.2 Preparation of Homogenate and Subcellular Fractions of Bovine Kidney

The cross-section of a mammalian kidney is shown in Fig. 3.1 [68]. A section through the organ shows its division into cortex, beneath the fibrous renal capsule, and medulla and both of them include anatomically and physiologically widely different structures, like nephrons, collecting tubules, blood vessels and others.

Samples of bovine kidney were purchased from a local supplier and the homogenate preparation as well as further fractionation were performed on the same day. The cortex and medulla tissues were cut up into small cubes, minced in an ice-cold isotonic sucrose buffer (0.25 M sucrose, 0.010 M HEPES, pH 7.4) in 1:4 ratio and blended for 5 s. The resulting tissue suspension was then centrifuged at 700xg for 10 min and the supernatant discarded; a fresh portion of the same volume of sucrose buffer with 0.010 M phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor was added and the homogenate was prepared using the Potter-Elvehjem glass homogenizer.

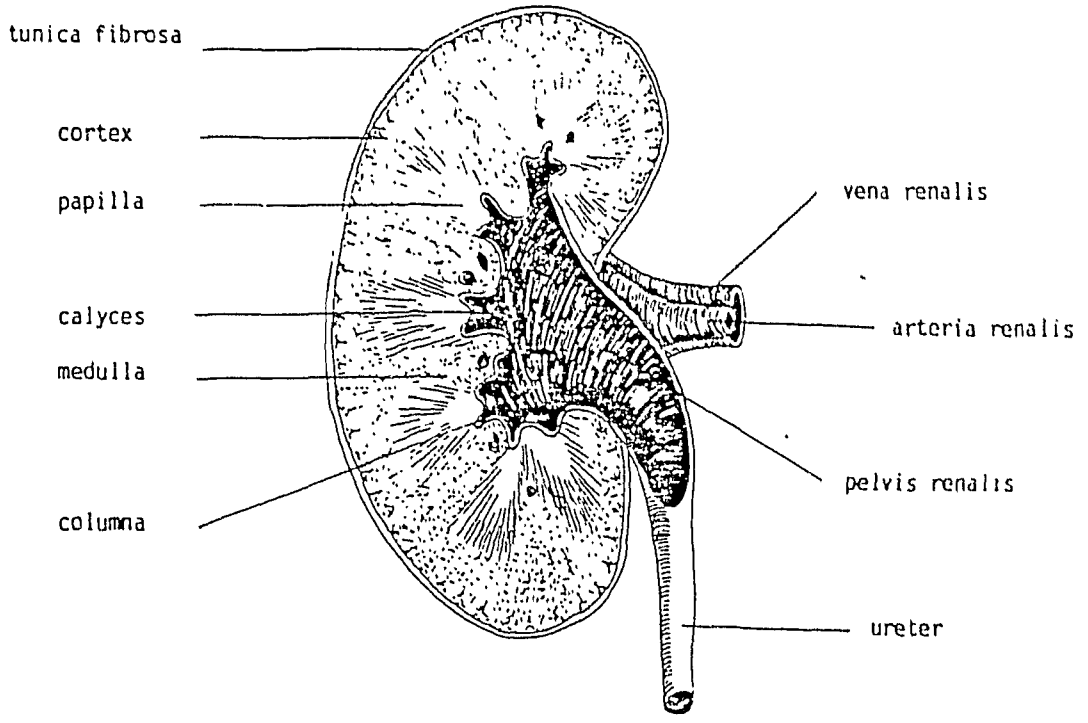


Fig. 3.1. Cross-section of a mammalian kidney [68].

The fractionation of cell components was carried out by means of differential centrifugation using a modified method of Sabbioni and Marafante [93], shown schematically in Fig. 3.2. First centrifugation was done at 700xg for 10 min to yield the nuclei fraction, then at 9 000xg for 10 min to obtain mitochondria and at 30 000xg for 25 min for lysosomes. Microsomes were separated from cytosol fraction by centrifugation at 100 000xg for 110 min. All centrifugations except the last one were carried out in a Sorvall RC-5B Refrigerated Superspeed Centrifuge with GSA rotor, r_{avg} = 9.17 cm or SS-34 rotor, r_{avg} = 4.25 cm, and the last one at 100 000xg was done using a Beckman Model L Ultracentrifuge with a type 50.2 Ti rotor, r_{avg} = 5.90 cm.

For the purpose of multielement analysis by neutron activation, samples of subcellular fractions were freeze-dried and known masses of powders were packed into irradiation vials.

3.2.3 Determination of Dry Mass

Dry mass was determined in a whole bovine kidney and in its subcellular fractions. A known amount of the fresh sample was placed in a precleaned plastic container of a known mass, frozen in liquid nitrogen, put into the freeze-drying glass flask and the flask attached to the Edwards Modulyo freeze dryer, connected to the Edwards E2M8 vacuum pump. After several hours, the dry sample along with the container was weighed, weight of the empty container subtracted and dry mass of the sample calculated. The resulting powders were stored in tightly

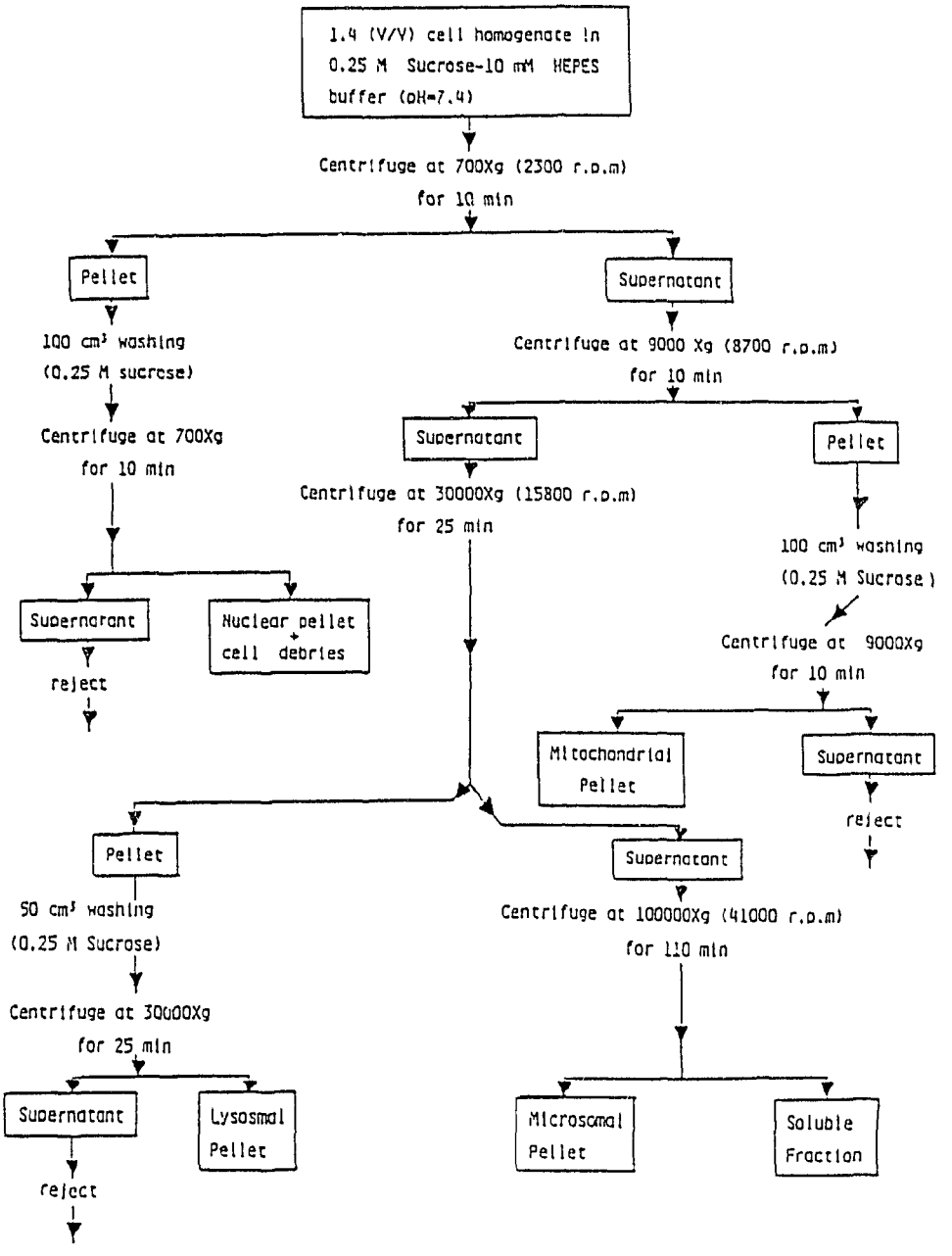


Fig. 3.2. Scheme for subcellular fractionation of bovine kidney.

closed acid-washed glass bottles at -20°C for further use.

3.2.4 Apparatus for Absorbance Measurements

A diode array spectrophotometer (Hewlett-Packard HP 8452A) with a 1 cm quartz cell and thermostated cell holder (if necessary) was used in this study to measure the absorbance of the reaction mixtures in all kinetic and colorimetric determinations. The spectrophotometer was interfaced to an IBM-compatible computer and the HP software was used.

3.2.5 Determination of Protein Content

Protein in the homogenate and subcellular fractions was assayed according to the method of Lowry *et al.* [94]. The determination is based on the Folin-Ciocalteu phenol reagent, the active constituent of which is phosphomolybdic-tungstic acid. Proteins reduce this mixed acid, producing one or more of several possible species which have a characteristic blue color. Copper, present in the reaction mixture, chelates in the peptide structure, facilitates the mechanism of acids reduction and increases the sensitivity to protein. Tyrosine and tryptophan and to a lesser extent cystine, cysteine and histidine are the principal amino acids taking part in the reaction. The absorbance measured at 650 nm is proportional to the protein concentration.

All samples were dialysed against water prior to analysis. A solution of crystalline bovine serum albumin (BSA, 98-99% albumin, essentially free of fatty

acids and globulin) was used as a protein standard.

3.2.6 Determination of Glutathione Peroxidase Activity

Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was determined using the method described by Paglia and Valentine [95]. The enzyme sample was mixed with an equal volume of Drabkin's reagent (diluted 1:1 with water) in order to convert hemoglobin to stable cyanmethemoglobin. An amount of 0.1 mL of this mixture was added to 2.58 mL phosphate buffer (50 mmol L^{-1}, pH 7.0, containing 5 mM EDTA). The following solutions were then added: 0.10 mL NADPH (8.4 mmol L^{-1}, tetrasodium salt, type I), 0.01 mL glutathione reductase (GSSG-R, A grade, yeast crystalline, 100 units per mg protein in 1 mL), 0.01 mL NaN_3 (1.125 mol L^{-1}) and 0.10 mL reduced glutathione (GSH, 0.15 mol L^{-1}, free acid, 98-100% crystalline). The reaction mixture was allowed to equilibrate at room temperature and then the enzymatic reaction was initiated by addition of 0.10 mL H_2O_2 (2.2 mmol L^{-1}, Fisher Scientific). The conversion of NADPH to NADP was followed by recording the change in absorbance at 340 nm every 30 s between 2 and 4 min after initiation of the reaction. The nonenzymatic oxidation of GSH was determined by replacing an enzyme solution by an equal volume of water in the reaction mixture. The reaction rate of this system was then subtracted from the one measured for the mixture containing an enzyme solution to determine the true enzymatic activity. Enzyme unit was expressed as micromoles of NADPH oxidized per min per mL of enzyme solution and was calculated on the basis of a

molar extinction coefficient of NADPH equal to $6.3x10^3$ L mol^{-1} cm^{-1}.

3.2.7 Evaluation of Purity of Subcellular Fractions Using Marker Enzymes

Cells exhibit complex structures when viewed under the microscope (Fig. 3.3) [96]. A number of discrete particulate components are visible including a nucleus, mitochondria, lysosomes, fat droplets, the Golgi substance, *etc.* The soluble materials comprise the remainder of the cell. Whether these soluble materials exist as such within the living cell or whether they are normally present in the subcellular structures and leak out during isolation is a question that is always difficult to answer. These cellular organelles exhibit distinct and unique functions in the living cell as they possess different substances and enzymatic apparatus located in them.

A method which allows one to study the intracellular distribution of proteins, especially enzymes and other substances within the cell, is the separation of tissue homogenate by means of differential centrifugation into particular subcellular fractions. The ideal separation procedure provides the isolated cell component with a high degree of purity as well as high yield. In the real experiment, however, errors can arise because of cross contaminations of the fractions and damage of the organelles due to careless handling.

Since some enzymes are located almost exclusively in one part of the cell, the measurement of the activity of a "marker enzyme" for a given subcellular fraction can be used for determining the purity of that fraction. This enzymatic

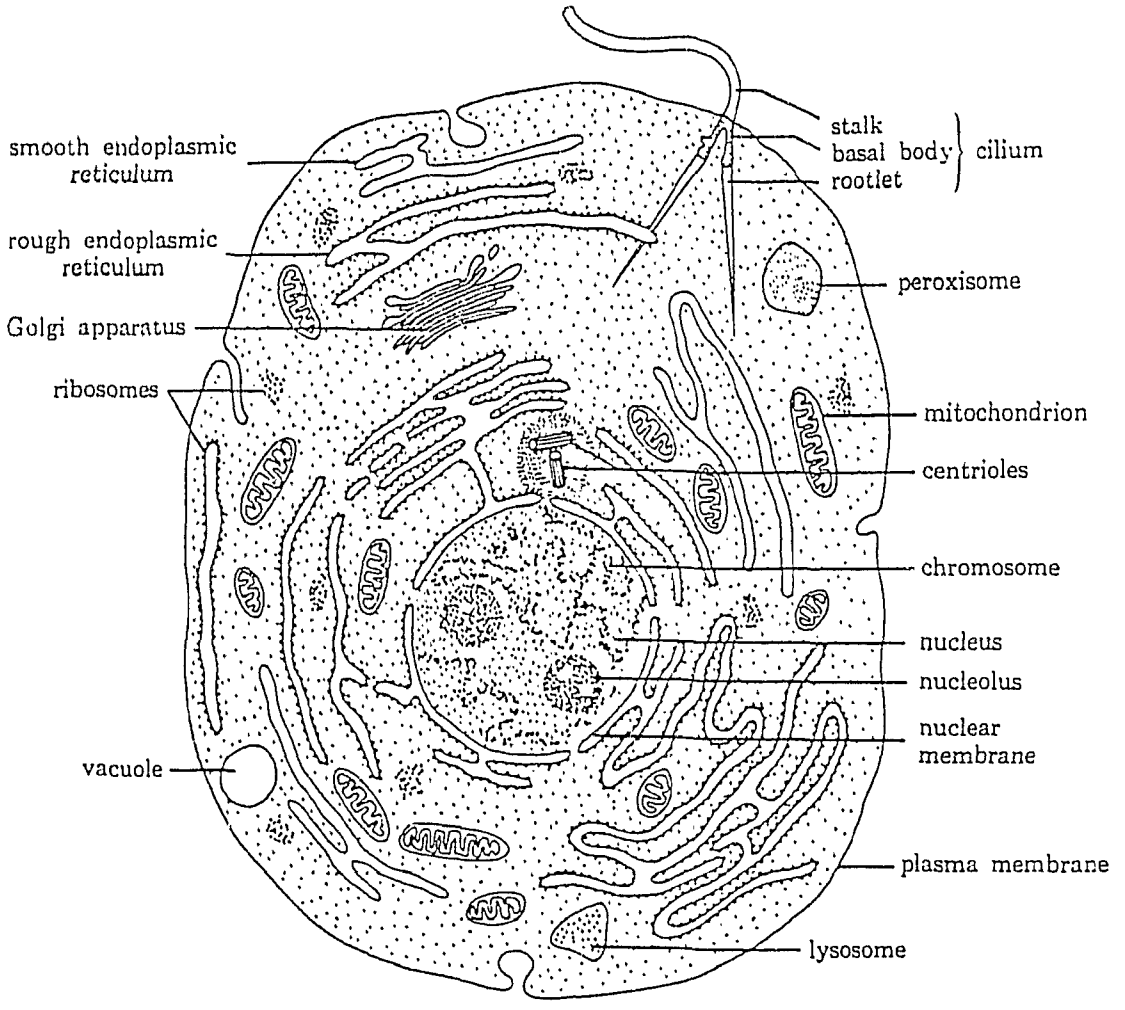


Fig. 3.3. A typical animal cell [96].

activity has to be assayed in each of the cell fractions and then the summed activity compared with that present in the initial homogenate, since the possibility of enzyme denaturation during the isolation procedure as well as the interaction with the inhibitors and activators used may have a remarkable influence on the results. The localization of a marker enzyme in a cellular component and thus the purity of the fraction can be indicated in three ways: (i) a large percentage of total activity of the homogenate (called the relative specific activity) is recovered in the fraction; (ii) the specific activity of the fraction is several times greater than that in the homogenate; and (iii) the specific activity of the fraction remains constant upon repeated separation.

A list of marker enzyme activities assayed in homogenate and all subcellular fractions is given in Table 3.1. The purity of the nuclear preparation was inferred from the absence of marker activities for other fractions.

Table 3.1

List of marker enzymes

Fraction	Marker Enzyme
Mitochondria	Glutamate dehydrogenase
Lysosomes	Acid phosphatase
Microsomes	Glucose-6-phosphatase
Cytosol	Lactate dehydrogenase

Glutamate Dehydrogenase (EC 1.4.1.3). Enzymatic activity was determined using a method described by Plummer [97]. The following aliquots were pipetted directly into a cuvette: 2.1 mL sodium phosphate buffer (0.1 mol L^{-1}, pH 7.4), 0.2 mL enzyme solution, 0.1 mL NADH (2.5 mg mL^{-1}, disodium salt, approx. 98%), 0.2 mL ammonium acetate (0.75 mol L^{-1}), 0.2 mL EDTA (30 mmol L^{-1}) and 0.1 mL 10% (w/v) Triton X-100 (BDH Chemicals). After a 10-min incubation at room temperature, 0.1 mL of sodium α-ketoglutarate (0.15 mol L^{-1}) was added and the rate of reaction was followed by reading the absorbance at 340 nm every minute for 5 min. Enzyme unit was expressed as nanomoles of NADH oxidized per min per mL of enzyme solution and was calculated on the basis of a molar extinction coefficient of NADH equal to $6.3x10^3$ L mol^{-1} cm^{-1}.

Acid Phosphatase (EC 3.1.3.2). The activity was measured according to the procedure given by Plummer [98]. Briefly, 0.2 mL of enzyme solution was mixed with 1.2 mL of the acetate buffer (0.2 mol L^{-1}, pH 4.5), 0.1 mL of 10% (w/v) Triton X-100 and 0.5 mL of the *p*-nitrophenyl phosphate (8 mmol L^{-1}). The mixture was then incubated for 10 min at room temperature and the reaction was stopped by adding 2 mL of the alkaline Tris buffer (Tris-HCl buffer, 1 mol L^{-1}, pH 9.0, containing 1 mol L^{-1} Na_2CO_3 and 0.4 mol L^{-1} K_2HPO_4). The absorbance at 405 nm was measured and *p*-nitrophenol was used to construct a standard curve.

Glucose-6-Phosphatase (EC 3.1.3.9). This assay was performed as described by Plummer [99]. The following mixture was equilibrated at room temperature for 10 min: 1.2 mL of sodium cacodylate buffer (0.1 mol L^{-1}, pH 6.5),

0.2 mL of EDTA (10 mmol L^{-1}) and 0.4 mL of glucose-6-phosphate (50 mmol L^{-1}). Enzymatic reaction was initiated by the addition of 0.2 mL of protein solution and stopped after 10 min by adding of 1.0 mL ice-cold 10% (w/v) trichloroacetic acid (TCA). The precipitate formed was removed by centrifugation and inorganic phosphate in the supernatant was determined colorimetrically at 880 nm. The sample consisted of 1 mL of supernatant, 3 mL of copper acetate buffer (2.5 g of copper sulfate and 46 g of sodium acetate in 1 L of 2 mol L^{-1} acetic acid, pH 4.0), 0.5 mL of ammonium molybdate (50 g L^{-1}) and 0.5 mL of reducing agent (20 g of *p*-methylaminophenol sulfate dissolved in a 100 g L^{-1} solution of sodium sulfite and made up to 1 L).

Lactate Dehydrogenase (EC 1.1.1.27). This enzyme activity was measured according to the method described by Vassault [100]. Briefly, 50 µL of enzyme solution was mixed thoroughly with 2.5 mL of Tris/NaCl/NADH solution (Tris, 81.3 mmol L^{-1}; NaCl, 203.2 mmol L^{-1}; NADH, 0.244 mmol L^{-1}, pH 7.2). The reaction was started at 30°C by the addition of 0.5 mL of Tris/NaCl/pyruvate solution (Tris, 81.3 mmol L^{-1}; NaCl, 203.2 mmol L^{-1}; pyruvate, 9.76 mmol L^{-1}, pH 7.2) and the change in absorption at 340 nm was recorded at 30 s intervals for 3 min. The enzyme unit was expressed as nanomoles of NADH oxidized per min per mL of enzyme solution and was calculated on the basis of a molar extinction coefficient of NADH equal to 6.3×10^3 L mol^{-1} cm^{-1}.

3.3 RESULTS AND DISCUSSION

3.3.1 Contaminants Present in Chemicals and Reagents

The only chemical used in large quantities in this part of the study was sucrose which was needed to prepare a buffer for subcellular fractionation. Inorganic contaminants present in solid sucrose were already discussed in the previous Chapter (Table 2.2). Only low levels of monovalent ions, *i.e.* Br^-, Cl^-, K^+, and Na^+, were detected and thus the 0.25 M sucrose buffer would contain approximately 2 ppb of Br, 70 ppb of Cl, 100 ppb of K and 25 ppb of Na. Since concentrations of these elements in kidneys are at least 1000 times higher (Table 3.2), it was concluded that none of them would interfere in biochemical studies on trace elements present in kidneys. Other steps taken in order to minimize the possibility of contamination are discussed in Section 2.4.1.1.6 of Chapter 2.

3.3.2 Elemental Content of Kidney and Its Subcellular Fractions

The major, minor and trace elements detected in a typical sample of whole bovine kidney are presented in Table 3.2. Because of the high levels of Na and Cl, concentrations of only 13 elements could be quantitively determined. Except for the studies done by Jayawickreme [70], no other investigations on trace element content of bovine kidneys have been found in the literature. Only studies done on rat and human kidneys were reported by others. Although it is understood that human, rat and bovine kidneys differ in terms of their elemental content, due to the lack of other references some comparisons of these data will be done at this

Table 3.2

Elements detected in bovine kidney[a]

Element	Concentration (ppm)[b]
Al[c]	0.71 ± 0.07
Br	3.51 ± 0.06
Cl	0.179 ± 0.003 (%)
Cu	2.58 ± 0.49
F	398 ± 4
Fe	74.7 ± 6.4
K	260 ± 4
Mg	164 ± 10
Mn	0.66 ± 0.08
Na	0.199 ± 0.002 (%)
Se[d]	1.43 ± 0.05
V	0.081 ± 0.015
Zn	21.9 ± 3.4

[a] average of 6 determinations;

[b] expressed as µg per g of fresh sample;

[c] corrected for P interference;

[d] determined using ^{77m}Se.

point.

A review of investigations done on the elemental composition of different fluids and organs of so called "reference man" is presented in a book by Heydron [73]. A "reference man" was defined in order to establish a reference frame of reliable data with which future results could be evaluated. Data for elements such as Mn, Cu, Zn, As, Se, Br, Rb, Mo and Ce are available. Concentrations of Br, Cu and Mn of a "reference man" are given as 3.9 ppm, 2.3 ppm and 0.5 ppm, respectively, and these values are comparable with our values (Table 3.2). Zinc seemed to be a difficult element to determine as its levels reported by various investigators differ considerably. The value given in a book [73] (60 ppm) exceeds three times the results of this work (21.9 ± 3.4 ppm) - this disagreement is most probably due to the differences in Zn content of human and bovine organs. The Se concentration given (0.75 ppm) is twice as low as the one found in this work (1.43 ± 0.05 ppm) and again data obtained by different researchers and using different techniques are not consistent.

Concentrations of trace elements in the cortex and medulla of rat kidney were studied by Beliveau *et al.* [101]. Only Al, Cu, Mn, Se, V, and Zn can be discussed because only these six elements were determined in both studies. The Se and Zn levels are comparable (Se: 1.43 ± 0.05 ppm in bovine kidney, 1.831 ± 0.114 ppm in rat kidney cortex, and 1.571 ± 0.302 ppm in rat kidney medulla; and Zn: 21.9 ± 3.4 ppm in bovine kidney, 19.223 ± 1.256 ppm in rat kidney cortex, and 20.147 ± 1.505 ppm in rat kidney medulla). Aluminum is about 4 times higher in

rats (3.545 ± 1.153 ppm in kidney cortex and 2.499 ± 0.839 ppm in kidney medulla) than in cow (0.71 ± 0.07 ppm). Copper is about 2 times higher in rat kidneys (6.255 ± 1.339 ppm in kidney cortex and 4.310 ± 0.363 ppm in medulla) than in bovine kidneys (2.58 ± 0.49 ppm). Manganese content is similar in bovine kidneys (0.66 ± 0.08 ppm) and in rat kidney cortex (0.783 ± 0.076 ppm), but not in medulla (1.281 ± 0.190 ppm). Vanadium level is comparable in cortex (0.100 ± 0.018 ppm), but twice that in medulla (0.174 ± 0.049 ppm) than in bovine kidneys (0.081 ± 0.015 ppm).

The percent distribution of 14 elements in subcellular fractions is shown in Table 3.3. Three samples of kidney were analyzed for this purpose and the results are presented as a range rather than an average ± standard deviation notation to give a better picture of the variability. It can be seen that the majority of elements were found in the nuclei fraction; the values ranged from about 40% for Al, Cu, Fe, Mg and Zn to about 50% for Br, Cr, F and V and up to 80% for Se. Mitochondria contained about 10% of Al, Fe and Zn, 15% of Mg, 20% of total Cu, 30% of Mn and 40% of total Cr and V. Up to 10% of Cu, Mg, Mn and Zn were detected in lysosomes which also had very high level of V (*ca.* 30%). The microsomal fraction was found to contain higher levels of only two elements: Al in quite broad range - from 2 to 13%, and about 15% of total Fe. Cytosol was very rich in monovalent ions such as Br^-, Cl^-, K^+, and Na^+ (up to 80% of their total content); it also contained up to 55% of total Fe, 40% of total Al, F and Zn, 20 - 40% of Mg, 30% of Mn 20% of Cu, 15% of Cr and 10% of Se.

Table 3.3

Distribution of trace elements in kidney subcellular fractions[a]

Element	Nuclei (%)	Mitochondria (%)	Lysosomes (%)	Microsomes (%)	Cytosol (%)
Al	42.2 - 50.9	5.3 - 16.7	6.0 - 7.4	2.1 - 13.0	23.3- 35.7
Br	30.2 - 55.8	2.0 - 6.3	0.5 - 1.1	0.4 - 1.0	57.3- 86.1
Cl	15.8 - 16.2	1.8 - 3.4	0.6 - 1.1	0.5 - 1.0	78.8-80.8
Cr	30.6 - 60.7	27.9 - 43.6	1.6 - 4.0	2.3 - 5.3	6.9 - 14.3
Cu	41.0 - 51.1	10.7 - 21.8	4.8 - 9.8	2.6 - 5.3	12.7-18.1
F	51.4 - 64.1	2.9 - 5.2	1.0 - 3.1	N/D	32.1-43.4
Fe	26.3 - 34.4	7.9 - 12.1	3.0 - 4.7	13.6 - 18.3	49.2-56.8
K	19.0 - 27.7	2.7 - 5.6	1.2 - 2.3	2.1 - 5.9	62.1-74.3
Mg	37.8 - 43.6	9.2 - 19.0	4.4 - 9.1	7.5 - 9.2	19.1-41.1
Mn	28.5 - 32.7	28.0 - 30.8	4.1 - 10.6	5.2 - 6.6	22.2-29.6
Na	18.6 - 21.3	3.3 - 6.8	1.3 - 3.8	1.3 - 1.8	67.6-75.0
Se	80.0 - 82.9	4.8 - 11.0	1.0 - 2.0	0.9 - 1.3	3.7 - 10.3
V	52.7 - 64.8	28.4 - 40.3	14.9 - 37.2	4.1 - 9.0	N/D
Zn	33.5 - 41.9	8.7 - 14.7	5.2 - 10.3	7.3 - 10.0	28.8-42.1

[a] average of 3 kidneys.

N/D = not detected.

These results can be compared with the data obtained previously in our laboratory by Jayawickreme [70]. Aluminum and F were not determined at that time so no comparison can be made. The percent distributions of Br, Cl, K and Na determined in all fractions are comparable. The values for all elements in the lysosomal and microsomal fractions also do not differ. Of all elements determined, the largest discrepancy was found in the case of V. In this work no V was detected in cytosol, whereas Jayawickreme [70] determined 37% of total V in this fraction. In the present study, the highest percentage of Cu was detected in the nuclei fraction, while by Jayawickreme [70] it was found in cytosol. Jayawickreme detected about 14% of total Se in the cytosol, while between 4% and 10% of Se was found in this fraction in the present work. Most likely, the differences in the results between the present and previous work are due to biological variations of trace elements in kidneys.

Protein contents of the homogenate and subcellular fractions of the three kidneys were measured and the results are presented in Table 3.4. The greatest variability in case of protein concentration was found in the homogenate (the relative standard deviation was calculated as ± 22%); the rest of the results were within ±15% of their mean values. The highest protein concentration was found in microsomes (121 mg mL^{-1}), yet this fraction contained the least amount of total protein, *i.e.* 4.2%. Cytosol had the lowest protein concentration (6.4 mg mL^{-1}) and contained 20% of the total protein. The nuclei fraction contained more than 50% of the total protein assayed.

Table 3.4

Protein concentration and distribution in bovine kidney subcellular fractions[a]

Fraction	Protein Concentration (mg mL^{-1})	Total Protein (g)	Distribution of Total Protein (%)
Homogenate	27 ± 6	11 ± 2	-
Nuclei	59 ± 6	5.0 ± 1.0	56
Mitochondria	66 ± 10	1.5 ± 0.3	14
Lysosomes	81 ± 3	0.5 ± 0.1	6
Microsomes	121 ± 10	0.4 ± 0.1	4
Cytosol	6.4 ± 1.0	2.0 ± 0.3	20

[a] average of 5 kidneys.

Total recovery: 90%.

Ratios of percent distribution of elements to percent distribution of proteins in a particular fraction are given in Table 3.5. Only Se can be considered as concentrated in the nuclei fraction with respect to the starting homogenate - the ratio calculated was 1.47. Chromium, Mn, and V got enriched in the mitochondrial fraction, the ratios being 2.5, 2.06 and 2.41, respectively. No significant enrichment of elements was found in the lysosomal fraction except for V for which the calculated ratio was as high as 4.5. Microsomes contained very little of total protein and several elements got concentrated in this fraction, ratios being 1.81 for Al, 3.81 for Fe, 2.0 for Mg, 1.4 for Mn, 1.57 for V and 2.07 for Zn. In the cytosol, for elements such as Br, Cl, F, K and Na which exist in cell fluids mainly as free ions, the ratios calculated were very high (3 and higher) except for F where the ratio was 1.88. Aluminum, Fe, Mg, and Zn were also found to be enriched in this fraction, the ratios being 1.47, 2.64, 1.50 and 1.77, respectively.

The above results can provide an useful information at the initial step of purification of metalloproteins. Although at this stage of investigations there is no evidence that the element of interest is bound to a protein, the highest ratios demonstrate which part of a cell accounts for most of the element of interest with respect to the protein content in that fraction. This observation may help to select the appropriate fraction for further studies of individual elements.

Table 3.5

Ratio of percent distribution of element to percent distribution of protein

Element	Nuclei	Mitochondria	Lysosomes	Microsomes	Cytosol
Al	0.84± 0.10	0.77± 0.41	1.16 ±0.19	1.81± 1.41	1.47± 0.35
Br	0.78±0.23	0.23± 0.12	0.14± 0.06	0.16± 0.08	3.57± -.83
Cl	0.288±0.001	0.18± 0.04	0.16± 0.06	0.19± 0.08	3.97± 0.47
Cr	0.82± 0.28	2.5 ± 0.61	0.48± 0.21	0.90± 0.44	0.53± 0.20
Cu	0.83± 0.11	1.14± 0.41	1.26± 0.46	0.95± 0.43	0.77± 0.16
F	1.04± 0.13	0.28± 0.08	0.34± 0.18	N/D	1.88± 0.36
Fe	0.55± 0.08	0.70± 0.16	0.67± 0.18	3.81± 1.23	2.64± 0.37
K	0.42± 0.08	0.29± 0.11	0.31± 0.11	0.95± 0.53	3.39± 0.50
Mg	0.73± 0.07	0.99± 0.36	1.17± 0.44	2.00± 0.61	1.50± 0.58
Mn	0.55±0.002	2.06± 0.24	1.28± 0.59	1.40± 0.43	1.29± 0.24
Na	0.36± 0.03	0.36± 0.13	0.45± 0.23	0.38± 0.13	3.55± 0.06
Se	1.47± 0.10	0.55± 0.22	0.26± 0.09	0.48± 0.14	0.35± 0.17
V	1.06± 0.13	2.41± 0.49	4.50± 2.00	1.57± 0.75	N/D
Zn	0.68± 0.08	0.82± 0.23	1.34± 0.48	2.07± 0.68	1.77± 0.39

N/D = not detected.

3.3.3 Determination of Dry Mass

Dry masses of five kidney samples were determined and the results are given in Table 3.6. No significant variability among different kidneys was observed. All results are within ± 10% of the mean values.

Table 3.6

Dry mass of homogenate and subcellular fractions[a]

Fraction	Dry Mass (%)
Homogenate	11.5 ± 1.0
Nuclei	13.9 ± 1.4
Mitochondria	14.9 ± 0.8
Lysosomes	18.1 ± 1.8
Microsomes	27.2 ± 2.0
Cytosol	7.9 ± 0.8

[a] average of 5 kidneys.

3.3.4 Glutathione Peroxidase Activity

Five different kidney samples were analyzed for glutathione peroxidase activity and the results are presented in Table 3.7. The highest enzyme activity was found in mitochondria (112±1 u mL^{-1}) but it constituted only 18% of the total

Table 3.7

Glutathione peroxidase activity in subcellular fractions of bovine kidney[a]

Fraction	Activity ($u\ mL^{-1}$)[b]	Specific Activity ($u\ mg^{-1}$protein)	RSA	Recovery of Activity (%)
Homogenate	41.8 ± 7.8	1.69 ± 0.16	(1)	(100)
Nuclei	70.2 ± 1.1	1.24 ± 0.10	0.74 ± 0.06	31.5 ± 1.7
Mitochondria	112.2 ± 1.0	1.67 ± 0.19	0.99 ± 0.10	18.1 ± 1.2
Lysosomes	67.5 ± 1.1	0.81 ± 0.14	0.48 ± 0.05	2.9 ± 0.2
Microsomes	80.2 ± 11.6	0.62 ± 0.09	0.37 ± 0.02	1.80 ± 0.05
Cytosol	23.4 ± 0.4	3.23 ± 0.08	1.91 ± 0.14	36.4 ± 2.0

[a] average of 5 kidneys.

[b] units defined as micromoles of NADPH oxidized per min.

Total recovery of enzyme 90.7 ± 7.2 %.

activity assayed. The highest specific activity was found in the cytosolic fraction (3.23±0.08) and the second highest specific activity (1.67±0.19) was determined in mitochondria. The highest relative specific activities were also found in these two fractions. These findings are in good agreement with earlier investigations of Motsenbocker and Tappel [43] who also found the highest enrichment of this enzyme in mitochondria and cytosol. Total recovery of an activity in all fractions was 90.7%. These data will be discussed in more detail in conjunction with the Se results in Chapter 4.

3.3.5 Marker Enzymes and Cross-Contamination of Subcellular Fractions

Four activities of marker enzymes have been assayed to determine the purity of kidney subcellular fractions and five different kidneys were analyzed for this purpose. The following activities were determined: glutamate dehydrogenase for mitochondria, acid phosphatase for lysosomes, glucose-6-phosphatase for microsomes and lactate dehydrogenase for the cytosol fraction. Separate data for the activity, specific activity and recovery of particular enzyme in each fraction are given in Tables 3.8 through 3.11, respectively.

A summary of the specific activities of all enzymes in all fractions is presented in Table 3.12. Poor precision observed was most likely due to variations of activities in different kidneys. Each enzyme became enriched in a fraction it represented except for acid phosphatase whose activity did not increase significantly in lysosomal fraction compared to that of the starting homogenate and

Table 3.8

Glutamate dehydrogenase activity in homogenate and subcellular fractions[a]

Fraction	Activity (u mL^{-1})[b]	Specific Activity (u mg^{-1}protein)	RSA	Recovery of Activity (%)
Homogenate	61.3 ± 11.4	2.26 ± 0.29	(1)	(100)
Nuclei	145.7 ± 37.5	2.60 ± 0.23	1.15 ± 0.18	48.0 ± 10.8
Mitochondria	320.2 ± 68.4	4.76 ± 1.18	2.11 ± 0.59	37.5 ± 3.8
Lysosomes	140.5 ± 11.5	1.74 ± 0.20	0.77 ± 0.13	3.7 ± 1.3
Microsomes	352.5 ± 61.8	3.26 ± 0.27	1.44 ± 0.22	4.5 ± 0.8
Cytosol	1.52 ± 0.30	0.27 ± 0.09	0.12 ± 0.04	1.4 ± 0.9

[a] average of 5 kidneys.

[b] units defined as nmoles of NADH oxidized per min.

Total recovery of enzyme 86.1 ± 11.6 %.

Table 3.9

Acid phosphatase activity in homogenate and subcellular fractions[a]

Fraction	Activity (u $mL^{-1}x10^2$)[b]	Specific Activity (u mg^{-1} protein $x10^4$)	RSA	Recovery of Activity (%)
Homogenate	1.67 ± 0.38	8.39 ± 1.51	(1)	(100)
Nuclei	4.53 ± 1.03	7.60 ± 0.79	0.91 ± 0.19	40.6 ± 4.3
Mitochondria	4.09 ± 1.67	5.33 ± 0.33	0.64 ± 0.12	10.7 ± 2.8
Lysosomes	6.73 ± 1.56	9.89 ± 1.14	1.18 ± 0.25	5.0 ± 1.0
Microsomes	13.30 ± 1.32	9.24 ± 0.98	1.10 ± 0.23	3.6 ± 1.1
Cytosol	0.31 ± 0.13	4.03 ± 0.62	0.48 ± 0.11	18.7 ± 1.0

[a] average of 5 kidneys.

[b] units defined as µmoles of PNP formed per min.

Total recovery of enzyme 78.6 ± 5.4 %.

Table 3.10

Glucose- 6 -phosphatase activity in homogenate and subcellular fractions[a]

Fraction	Activity (u $mL^{-1}x10^5$)[b]	Specific Activity (u mg^{-1} protein $x10^6$)	RSA	Recovery of Activity (%)
Homogenate	4.20 ± 1.45	1.62 ± 0.63	(1)	(100)
Nuclei	6.36 ± 1.84	1.37 ± 0.18	0.84 ± 0.34	36.8 ± 6.0
Mitochondria	7.15 ± 0.82	1.17 ± 0.12	0.72 ± 0.29	15.0 ± 1.1
Lysosomes	56.6 ± 7.4	7.14 ± 0.86	4.41 ± 1.80	25.5 ± 3.1
Microsomes	58.0 ± 16.6	4.75 ± 0.62	2.93 ± 1.20	17.5 ± 2.8
Cytosol	0.05 ± 0.01	0.12 ± 0.01	0.07 ± 0.03	1.5 ± 0.1

[a] average of 5 kidneys.

[b] units defined as mmoles of glucose-6-phosphate hydrolysed per min.

Total recovery of enzyme 96.3 ± 7.4 %.

Table 3.11

Lactate dehydrogenase activity in kidney and subcellular fractions[a]

Fraction	Activity (u mL^{-1})[b]	Specific Activity (u mg^{-1}protein)	RSA	Recovery of Activity (%)
Homogenate	91.0 ± 3.2	3.81 ± 0.46	(1)	(100)
Nuclei	82.4 ± 10.1	1.41 ± 0.22	0.37 ± 0.07	18.2 ± 0.9
Mitochondria	75.3 ± 8.4	0.86 ± 0.17	0.21 ± 0.01	3.6 ± 0.9
Lysosomes	84.0 ± 12.0	1.15 ± 0.24	0.30 ± 0.07	1.7 ± 0.2
Microsomes	56.2 ± 6.4	0.40 ± 0.06	0.10 ± 0.02	1.0 ± 0.3
Cytosol	109.6 ± 5.6	20.15 ± 2.76	5.29 ± 0.97	76.0 ± 5.1

[a] average of 5 kidneys.

[b] units defined as nmoles of NADH oxidized per min.

Total recovery of enzyme 100.5 ± 5.3 %.

Table 3.12

Relative specific activities (RSA)[a] of marker enzymes in subcellular fractions[b]

Subcellular Fraction	Glutamate Dehydrogenase	Acid Phosphatase	Glucose-6-Phosphatase	Lactate Dehydrogenase
Nuclei	1.15 ± 0.18	0.91 ± 0.19	0.84 ± 0.34	0.37 ± 0.07
Mitochondria	2.11 ± 0.59	0.64 ± 0.12	0.72 ± 0.29	0.21 ± 0.01
Lysosomes	0.77 ± 0.13	1.18 ± 0 25	4.41 ± 1.80	0.30 ± 0.07
Microsomes	1.44 ± 0.22	1.10 ± 0.23	2.93 ± 1.20	0.10 ± 0.02
Cytosol	0.12 ± 0.04	0.48 ± 0.11	0.07 ± 0.03	5.29 ± 0.97

[a] RSA expressed as ratio of specific activity of an enzyme in a fraction to the specific activity of this enzyme in homogenate.

[b] an average of three kidneys.

other fractions. Nuclei appeared as being highly contaminated since relative specific activities of the first three enzymes equalled to about 1; only the activity of lactate dehydrogenase diminished by about 60%. There is also a great possibility of cross-contamination among mitochondrial, lysosomal and microsomal fractions as indicated by results for the first three enzymes. The activity of glucose-6-phosphatase in lysosomes even exceeded the enzyme activity in microsomes, the values being 4.41 (lysosomes) and 2.93 (microsomes).

Although the most likely reason for the above results is the cross-contamination of different fractions, one has to bear in mind that none of the enzymes assayed is present exclusively in one fraction - they are said to be present predominantly in one fraction or in the other. Different researchers come to contradictory conclusions on this topic. For example, according to Tolbert [102], lactate dehydrogenase is not a satisfactory enzyme as a cytosol marker because several percent of this activity is present in fractions containing microsomes and mitochondria. However, this is the most widely applied assay for the determination of the purity of the cytosol fraction. A very detailed study on the intracellular distribution of enzymes in rat liver was done by De Duve *et al.* [103]. It was stressed by the authors that an enzyme could denature during fractionation and that some argumentative results might be due to the difficulties in assaying the enzymatic activity in complex media.

The results obtained for the cytosol (last row of Table 3.12) indicated that the fraction was fairly free from activities of other enzymes (maximum of 20% of

contaminating activity present) and also it was rich in activity of its marker enzyme (specific activity of lactate dehydrogenase increased by 5.3-fold). It was concluded that the purity of this fraction was satisfactory for further studies on protein-bound trace elements.

3.4 SUMMARY

The concentrations of 14 major and trace elements in bovine kidney and its subcellular fractions were determined using INAA. High content of Na and Cl did not allow for the determination of elements such as Ag, As, Cd, Cr, Hg, Mo, Pb, Rb, S, Sb, Sn and W which are either present at very low levels or are insensitive by INAA. Differential centrifugation was applied to fractionate trace elements according to their subcellular distribution. The degree of elemental enrichment was investigated by calculating the ratio of percent distribution of an element to the percent distribution of protein in a particular fraction.

The purity of cell subfractions was evaluated by determining the activities of four marker enzymes; possible cross-contamination was observed among mitochondrial, lysosomal and microsomal fractions. For further studies on these fractions the fractionation procedure needs to be improved in order to minimize the possibility of contamination. Cytosol, on the other hand, turned out to be fairly free from activities of other marker enzymes and rich in the activity of lactate dehydrogenase which is the cytosolic marker. Studies on this fraction were therefore continued without any further purification.

4. PURIFICATION OF SELENOPROTEINS IN CYTOSOL FRACTION

4.1 INTRODUCTION

The main objective of the work presented in this chapter was to develop analytical and bioanalytical methods for investigating Se-binding proteins in the cytosol fraction of bovine kidneys. The majority of the studies on animal selenoproteins involves either injecting animals with radioactive ^{75}Se or feeding them overdoses of Se in diet. Under these conditions erroneous results may be obtained due to two reasons: (i) a radiotracer might not get fully equilibrated with the Se present in the body; and (ii) an excess of Se from a diet might randomly bind to proteins. The samples analyzed in these studies come form animals fed regular diets and hence the Se level and Se-proteins detected can be considered as existing in this organ under natural conditions.

The first part of this chapter gives an extensive literature survey done on the present state of knowledge of Se and selenoproteins. In the second part, the experiments that have been performed and results obtained are discussed.

4.1.1 Toxicity and Essentiality of Selenium

Selenium, a metalloid (atomic number 34) chemically related to S, was discovered by Berzelius in 1818 when he was searching for a source of Te in sulfuric acid [104]. It is a naturally occurring element found in rock, sandstone,

limestone, coal, soil, surface water and vegetation [105].

Selenium is now placed among vital micronutrients. However, long before it was considered essential, Se was well-known for its toxicity. The first recognition that Se had any biological activity came in 1936 when it was found to be the principal toxin associated with neuropathies and dermatopathologies of grazing horses and cattle in the northern Great Plains [105]. Species of Se-accumulator plants can contain extremely high levels of Se in the form of nonprotein selenoamino acids such as Se-methyl-selenocysteine and can cause acute toxic reactions in grazing animals. The "blind staggers" disease is a subacute selenosis which occurs in livestock consuming plants containing 100 - 10 000 ppm Se. This disease is characterized by neurologic deterioration (blindness) and disorientation with generalized paralysis in the final stage and death being due to respiratory failure [106]. A chronic form of selenosis, an alkali disease, takes place among livestock feeding on plants containing 20 - 50 ppm Se. Its major symptoms are emaciation and dermatologic changes such as alopecia and hoof necrosis [106].

Selenium poisoning has been induced in laboratory animals using various routes of administration. The dose of Se ingestion that can cause selenosis depends on the chemical form of Se, the presence of other components in the diet and the time involved in consuming Se-rich foodstuffs [104]. The most toxic form of Se is hydrogen selenide but apart from industrial exposures poisoning by this compound occurs very rarely. The toxicities of selenite and selenate are similar,

and selenite and selenomethionine have also similar toxicities when administrated over long periods of time. It was observed by Parizek *et al.* [107] that methylated Se compounds were much more toxic towards male rats in comparison to females. The most important factors that protect against Se poisoning are high protein content of a diet (due to the presence of methionine and S) and vitamin E supplementation. The toxicity of Se can be altered by interaction with heavy metals such as As, Cd, Cu, Pb, Hg, Ag and Zn [105].

Selenium-induced problems in human health generally result from unusual situations. Human Se toxicosis *via* inhalation or dermal contact has been reported from industrial and other accidental exposures [108]. Poisoning by Se-containing products *via* gastrointestinal track occurs rarely - it is usually due to a consumption of mislabelled superpotent selenium pills or other mistaken foodstuff. In Venezuela, a case of acute selenosis after ingestion of "coco de mono" nuts containing high amount of Se has been noted; the patient eventually recovered completely [109].

An endemic chronic Se intoxication characterized by a loss of hair and nails, skin lesions and neurologic changes was recognized in China in 1961 [23] where the element became available for crop uptake by weathering off to the soil from a stony coal extremely rich in Se (90 000 ppm).

Experiments performed on laboratory rats and rabbits have shown cataractogenicity and cariogenicity of Se [24]. An example of chronic selenosis from China mentioned above [23] where increased incidence of dental caries took

place suggest that humans might be prone to the cariogenic effects of Se as well.

Increasing knowledge of the high toxicity of Se led to serious concern about the possible carcinogenicity of this element. An interpretation of the experimental data on animals, however, could not give a direct positive answer [110]. Human epidemiologic studies also have not proven that Se is carcinogenic. For example, an inverse correlation has been reported between the number of cancer deaths in humans and the regions of the world with Se-rich soil [111], [112]. To date, the International Agency for Research against Cancer does not recognize Se and its compounds as carcinogenic in man [113].

In 1957, the understanding of the function of Se for living organisms changed drastically when Schwartz discovered its protective role against liver necrosis in laboratory rats [10]. This was the first step in the recognition that a number of previously unexplained metabolic disorders in animals and birds, *e.g.*, white muscle disease in cattle and sheep, pancreatic atrophy in chickens, mulberry heart in swine, are Se-responsive. In a review article published in 1983 [114] as many as 22 diseases are mentioned as related to low Se status.

Anticarcinogenic effects of Se have been reported in experimental animals where the element had a protective action against a variety of chemically and virally induced tumors [17], [115]. The levels of Se used in these studies were very high (5 ppm) and nearly produced acute toxicosis in the animals. In epidemiological studies, several cases revealed that patients with gastrointestinal cancers had lower blood levels of Se than controls [116] and these results were

confirmed later by McConnell *et al.* [117] and other researchers. The mechanisms of the anticarcinogenic properties of Se are uncertain; they may include effects on the oxidation of intracellular membranes, carcinogen metabolism, DNA repair and the influence on the immune system. Nevertheless, the possible use of Se as an anticarcinogenic agent in chemotherapy has been suggested by Hocman [118].

A connection between low Se status and increased incidence of cardiovascular diseases has also been postulated [119]. Animal experiments suggest that the Se deficiency changes arachidonic acid metabolism by increasing the production of thromboxanes (which promote platelet aggregation) and decreasing the production of prostocyclins (which are anti-aggregatory). This hypothesis is supported by the findings that there is a marked decrease in GSH-Px activity in platelets in Se-deficient state in rats and by the findings of altered metabolism of arachidonic acid metabolites in Se-deficient rats [120] and in Se-depleted humans [121].

Evidence for the nutritional importance of Se in man has come from Asia where two endemic Se-responsive conditions have been reported. Keshan disease is a type of cardiomyopathy affecting children and women of child-bearing age living in a rural area of China where the Se level of soil is low [122]. Its occurrence has been invariably associated with a low Se levels in hair and blood and a low GSH-Px activity. Addition of Se to the diet increased both Se level and the enzyme activity in blood. Selenium supplementation in controlled studies resulted in a 5- to 10-fold reduction in the incidence of this disease [12]. Kashin-

Beck disease is an endemic osteoarthropathy in children in Se-poor regions of northern China, North Korea and eastern Siberia [122]; in this case Se supplementation has also been shown to exhibit protective effects [123].

Most recently, an extremely low Se content in the soil and grain of two provinces of the former Yugoslavia has been linked to the existence of Balkan endemic nephropathy on these territories [124]. Serum selenium levels of residents from those areas are lower than in any other country in Europe [125] and Se content of grain and human scalp hair are approaching those in the Se-deficient parts of China.

So far, Se inadequacy is the only observed common denominator for all endemic areas and to date there is no convincing evidence that this deficiency alone is the primary cause of all these diseases.

4.1.2 Interaction of Selenium with Heavy Metals

Selenium is considered to be very reactive among the trace elements. Reactions involving Se compounds are very fast; for example, selenates (Se^{6+}) and selenites (Se^{4+}) are readily reduced in the animal body to the active selenide form (Se^{2-}). Selenium species are capable of exhibiting antagonistic effects in its interaction with some heavy metals such as Hg, Cd and Pb where the toxicity of both are altered.

Experiments on laboratory animals indicate that under most circumstances Se does not protect against heavy metal toxicity by increasing their excretion from

the body. Instead, the general mechanism of protection is an accumulation of metals in tissues as a result of Se intake. The diversion of metal binding by Se, as observed by Parizek *et al.* [126] and others, is one of the possible mechanisms involved in this protection. Selenium has been shown to divert the binding of Hg in kidney cortex from low MW proteins to high MW ones [127], [128] and it has been proposed that it is the selenide form of Se which takes part in this action. In addition, selenide could also react with metals to form insoluble metal selenides leading to reduced metal toxicity

The interaction of Se and methylmercury in brain is of particular interest. Despite the clear protective effect of selenite on methylmercury toxicity, the Hg content of brain increased in presence of Se supplementation [129], [130]. The protective role of Se could take place *via* the reaction of methylmercury with the selenohydryl group during which the less toxic methylmercury derivatives are formed. This type of mechanism seems to be possible since the great percentage of Se in animal tissues is present in the form of selenocysteine [105].

With respect to Cd which is a highly toxic heavy metal, Se has been shown to prevent or reduce various aspects of Cd toxicity including cadmium-induced lethality [131] and testicular necrosis [132]. As in case of Se-Hg interactions, Se in the form of selenide protected against cadmium toxicity by diverting the binding of injected Cd from low MW proteins to high MW proteins. In an investigation of Se and selenoproteins in rat kidney, Motsenbocker and Tappel [43] performed a dual-labelling experiment with ^{75}Se and ^{109}Cd in order to investigate whether rat

kidney selenoproteins can incorporate Cd in cases of acute Cd intoxication. It was found that up to 72 h after Cd administration no ^{109}Cd was associated with Se-containing proteins. The role of Se in inducing protection against Cd toxicity in monkeys was examined by Sidhu *et al.* [133]. The excessive Cd administration drastically lowered the activity of glutathione peroxidase and glutathione-S-transferase in organs such as liver, kidney, brain and lungs. Selenium administration immediately increased these activities back to the initial levels and simultaneous administration of both elements resulted in an increase in total activities of both enzymes. The mechanism by which Se decreases Cd toxicity in Rhesus monkeys seemed to rely on the protection of the enzyme systems, possibly by forming non-toxic Cd selenide.

As already mentioned, heavy metals influence the toxicity and metabolism of Se itself. In general, dietary supplementation of Ag, Cu, Hg or Cd to animals has been shown to reduce toxicity of Se [105]. The effect in most cases is an increase of Se uptake and retention in body tissues and changes in Se distribution of subcellular fractions. For example, methylmercury caused a very marked uptake of Se in brain but not in liver, kidney or muscle [134]. In brain, Se shifted from cytosol to mitochondrial fraction. Following Ag administration, the proportion of total Se found in liver mitochondria increased with a corresponding decrease in soluble and microsomal fractions of this tissue [135]. Parizek *et al.* [107] found that the toxicity of dimethylselenide in rats was increased synergistically with mercuric chloride for reasons unexplained.

4.1.3 Selenium and Sulfur - Similarities and Differences

Selenium and sulfur both exhibit the same oxidation states, namely 2-, 0, 4+ and 6+. Some of their physical and chemical properties such as the bond energies, ionization potentials, electronegativities and polarizabilities are very similar. However, the following reaction exemplifies why these two elements cannot always undergo *in vivo* substitution:

$$H_2SeO_3 + 2H_2SO_3 \text{ ----> } Se + 2H_2SO_4 + H_2O$$

The quadrivalent Se in selenite has a tendency to undergo reduction, whereas the quadrivalent S in sulfite tends to undergo oxidation. Thus, Se compounds tend to be metabolized in animals to more reduced states whereas S compounds incline to be oxidized.

The second difference is in the relative acid strengths of their hydrides. Although the analogous oxyacids of Se and S have comparable strengths, the hydride H_2Se (pK_a 3.8) is a much stronger acid than H_2S (pK_a 7.0). This difference is important in the dissociation behaviors of the selenohydryl group in selenocysteine (pK_a 5.24) and the sulfhydryl group of cysteine (pK_a 8.25). Thus, whereas thiols such as cysteine are mainly protonated at physiological pH, the selenohydryl groups of selenols such as selenocysteine are largely dissociated [105].

Animals, bacteria and possibly higher plants all require trace amounts of Se

which is then incorporated in a specific fashion into certain functional proteins of the cell. However, if the organism receives more Se than needed, those enzymatic systems which cannot distinguish Se from S begin to substitute Se indiscriminately for S [136]. A greater reactivity and lower stability of Se compounds compared to their S analogues may account for the toxicity of Se when this random substitution takes place. There are enzymes that selectively react with Se compounds at extremely low Se concentrations and serve as catalysts in normal Se metabolism. They have to be differentiated from the systems that do not discriminate between Se and S at elevated Se levels and are capable of producing appreciable amounts of the Se metabolite *in vivo*. Enzymes utilizing both S and Se compounds as substrates include biocatalysts that react with inorganic S and those involved in the metabolism of S amino acids. Examples listed by Stadtman [136] include: (i) ATP sulfurylase; (ii) cysteinyl-tRNA synthetase; (iii) methionyl-tRNA synthetase; (iv) tRNA sulfur transferase; (v) amino acid polymerase; (vi) S-adenosylmethionine synthetase; and (vii) S-adenosylmethionine methyl transferase.

Little is known at present about the specific effects of the replacement of S amino acids in proteins by their Se analogues. Clearly, the substitution of a selenocysteine or a selenomethionine residue for its S analogue at the active site of an enzyme or at positions important for the maintenance of the tertiary structure of a protein could be of much greater significance than if such replacement occurred in less critical regions. An interesting example of an enzyme that can

tolerate extensive replacement of methionine by selenomethionine and at the same time does not lose its catalytic activity significantly is the β -galactosidase from *E.coli* [137], [138].

4.1.4 Low-Molecular-Weight Organoselenium Compounds

It has already been mentioned that Se can exist in four oxidation states, *viz.* selenide (2-), elemental selenium (0), selenite (4+) and selenate (6+). The Se^{6+} is the form in which the element exists in inorganic salts, whereas Se^{2-} is the most important oxidation state of Se in organic compounds. Organoselenium molecules can be further divided into compounds of low- and high molecular weights.

There are several low-MW organoselenium species that occur naturally in living systems. Shamberger [104] lists them as follows:

(i) Selenocysteine, $HSe\text{-}CH_2CH(NH_2)COOH$, found in protein hydrolysates of corn, wheat seeds, sheep wool, in some prokaryotic and eukaryotic enzymes including glutathione peroxidase.

(ii) Selenocystine, $HOOCCH(NH_2)CH_2\text{-}Se\text{-}Se\text{-}CH_2CH(NH_2)COOH$, found in rye grass, red and white clover, onion and also in some enzymes.

(iii) Selenohomocystine, $HOOCCH(NH_2)(CH_2)_2\text{-}Se\text{-}Se\text{-}(CH_2)_2CH(NH_2)COOH$, found in the leaves of the Se-accumulator plant *Astragalus crotalariae*.

(iv) Se-methylselenocysteine, $CH_3\text{-}Se\text{-}CH_2CH(NH_2)COOH$, which is 80% of the total selenium content of the other Se-accumulator plant *Astragalus bisulcatus*.

(v) Selenocystathione, $HOOCCH(NH_2)CH_2\text{-}Se\text{-}CH_2CH_2CH(NH_2)COOH$, found

in small quantities in the plant *Astragalus pectinatus* and it has also been identified as the cytotoxic compound in the seeds of "coco de mono" nuts grown in South America.

(vi) Selenomethionine, CH_3-Se-$CH_2CH_2CH(NH_2)COOH$, detected in β - galactosidase from *E.coli* grown in a high selenate and low sulfate medium where it replaced an amino acid methionine [137], [138].

(vii) Se-methylselenomethionine, $[(CH_3)_2$-Se-CH_2CH_2-$CH(NH_2)COOH]^+$, detected in clover and rye grass roots and also as the predominant organoselenium compound synthesized from selenite by non-selenium accumulator species of *Astragalus*.

(viii) Dimethyl selenide, CH_3-Se-CH_3, isolated from *Aspergillus niger* and *Scopulariopis brevicaulis* grown with selenite and in *Astragalus* plants and seeds. This organoselenium compound was also detected as a respiratory product in rats loaded with selenite.

(ix) Dimethyl diselenide, CH_3-Se-Se-CH_3, a volatile compound identified in *Astragalus racemosus*.

(x) Trimethylselenonium ion, $(CH_3)_3Se^+$, a metabolic product excreted by the urinary system.

(xi) selenotaurine, $H_2NCH_2CH_2$-SeO_3H, found as a derivative of bile cholic acid in sheep injected with $Na_2{}^{75}SeO_3$.

4.1.5 Glutathione Peroxidase (GSH-PX)

Glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9) was first described in 1957 by Mills [139] who found that it prevents oxidation of hemoglobin in erythrocytes and protects erythrocytes from haemolysis induced *in vitro* by H_2O_2. Subsequent work of Little [140] and others demonstrated that GSH-Px can also catalyze the reduction of lipid or sterol hydroperoxides according to the following general reaction:

$$ROOH + 2GSH \xrightarrow{\text{GSH-Px}} ROH + H_2O + GSSG$$

where R: lipid, sterol or H.

Studies by Rotruck *et al.* [11] in 1973 on the effects of dietary Se on oxidative damage of rat erythrocytes led to the discovery of a Se-dependent red blood cell (RBC) GSH-Px activity. This GSH-Px has been purified from several tissues of a number of species and was found to be localized in the cytosol and mitochondria [105]. This is a tetrameric enzyme with a molecular weight (MW) of approximately 80 kDa depending on the tissue from which it was isolated and containing 1g-atom of Se per subunit in the form of a single selenocysteine molecule. Neutron activation analysis of crystalline enzyme did not detect any other metals beside Se [141]. Amino acid analysis of rat liver GSH-Px showed that each subunit consists of two cysteine, three methionine and thirteen aromatic residues out of a total of 153 amino acids [105].

The Se-GSH-PX enzyme has a high specificity for its donor substrate, reduced glutathione (GSH), but is much less specific for acceptor substrates. Various lipid hydroperoxides and nucleic acid hydroperoxides are reduced by this enzyme; however, it does not reduce esters of fatty acid hydroperoxides which are expected to be present in biological membranes.

Apart from the cellular glutathione peroxidase (c-GSH-Px) isolated by Rotruck *et al.* [11] three other forms of Se-dependent GSH-Px in the blood and organs of mammals have been recognized so far: (i) an extracellular or plasma form (p-GSH-Px); (iii) phospholipid hydroperoxide (PHGSH-Px); and (iii) GSH-Px-GI, respectively.

Like c-GSH-Px, p-GSH-Px is also a tetrameric enzyme containing 1g atom of Se per subunit [142]. The plasma form is glycosylated, is more temperature-stable, has a lower specific activity and a higher subunit molecular weight than the cellular form [143]. Polyclonal antibodies produced against one form do not cross-react with the other form of the enzyme [144]. The above immunologic studies also demonstrated that only a small fraction of Se in plasma and RBCs of healthy subjects is associated with GSH-Px. These findings imply that blood glutathione peroxidase activity cannot serve as an index of Se status in humans. Avissar *et al.* [145] reported that the majority of GSH-Px activity in human milk is due to p-GSH-Px.

The PHGSH-Px enzyme is a 18-kDa monomer and has different substrate specificities compared to c-GSH-Px and p-GSH-Px [146]. This enzyme catalyzes

the reduction of phospholipid hydroperoxide, cholesterol hydroperoxide and linoleic acid hydroperoxide much more effectively than the reduction of H_2O_2 and tert-butyl hydroperoxide.

The most recently discovered member of the glutathione peroxidase family is GSH-Px-GI [147]. This is a tetrameric enzyme composed of 22-kDa monomers localized in cytosol but not in the particulate fractions of the cell. It has relatively higher reactivity towards organic hydroperoxides compared to H_2O_2. It was detected predominantly in human and rat gastrointestinal (GI) tract from which its name was derived.

4.1.6 Glutathione Peroxidase, Vitamin E and Other Antioxidants

The first demonstration of the essentiality of Se as an integral part of Factor 3 in the prevention of liver necrosis in rats revealed relationship of Se to the biochemical function of vitamin E. Now it is known that Se-GSH-Px functions as part of a multi-component defense system within the cell and protects membrane lipids, proteins and nucleic acids against oxidant damage. Hydrogen peroxide, hydroperoxides, superoxide, various radicals including hydroxy radical and possibly singlet oxygen are formed as products and/or byproducts of necessary reactions in cells and they have to be destroyed by the protective mechanisms before they damage the cells. These protective systems are compartmentalized and thus complement one another. For example, both GSH-Px and catalase can reduce H_2O_2 but in most cells these enzymes exist in distinct subcellular fractions

(catalase in the peroxisomes and GSH-Px mainly in the cytosol and mitochondrial matrix space), so there is little competition of these enzymes for metabolically produced H_2O_2. As a lipid-soluble antioxidant, vitamin E scavenges free radicals before they can attack cellular and intracellular membranes; if GSH-Px does not destroy the peroxides then vitamin E can still protect the membrane. Superoxide dismutase (Cu and Zn-dependent) destroys cytosolic and mitochondrial superoxides before they can react with H_2O_2 to form hydroxy radical [105]. The interrelationships among Se, vitamin E and sulfur amino acids are shown schematically in Fig. 4.1 [105].

Most recently, Zamora *et al.* [148] studied the effectiveness of other compounds in protecting red blood cells and plasma against oxidative damage. They found that β-carotene and coenzyme Q_{10} were almost as active antioxidants as Se and vitamin E.

4.1.7 Selenium, and Selenoproteins Other than Glutathione Peroxidase

For several years, the only known selenoprotein with well-established function in mammalian and avian tissues was glutathione peroxidase. In recent years, however, a number of biological activities such as prevention of hepatic heme degradation, compensation for vitamin E and maintenance of plasma glutathione level were found not to be associated with this enzyme but yet dependent on Se status [149]. These inconsistencies prompted investigators to search for other Se-binding proteins in order to further explore the biochemical and

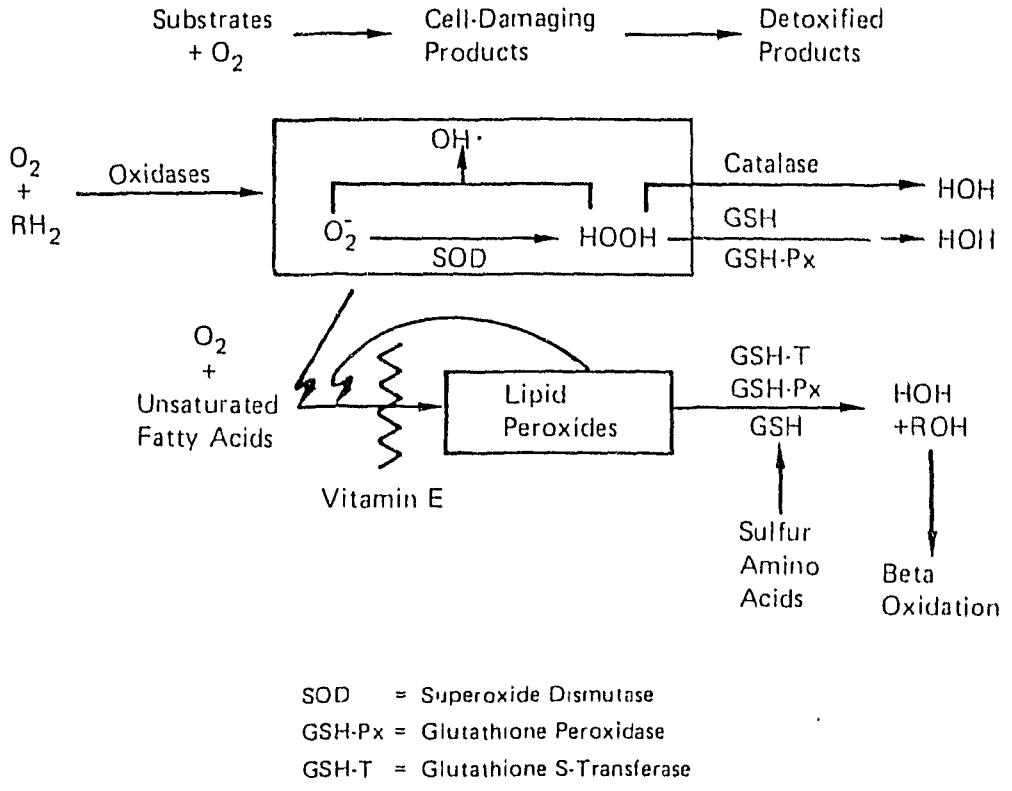


Fig. 4.1. Interrelationships among selenium, vitamin E and sulfur amino acids [105].

physiological role of Se.

Investigations started with the determination of the total Se level in various tissues and fluids as this could denote some specific Se target organs in the body. Behne *et al.* [32] studied rats fed with Se in a diet. The highest concentration of the element was detected in kidneys (1.45 ± 0.25 ppm), liver (1.29 ± 0.03 ppm) and testes (0.89 ± 0.05 ppm), and the lowest in brain (0.13 ± 0.01 ppm), muscle (0.16 ± 0.01 ppm) and heart (0.37 ± 0.04 ppm), as calculated per wet mass. A similar Se tissue distribution was found by Hawkes *et al.* [31] who also determined the concentration of Se in plasma as 0.6 ppm. Within the liver of rats injected with [^{75}Se]selenite [150], the highest radioactivity was found in the cytosol fraction followed by mitochondria, microsomes and nuclei. In rat kidneys, the highest enrichment of Se over that of the starting homogenate was found in lysosomes, and next in mitochondria and cytosol [43]. On the other hand, bovine kidneys were found to contain around 2.55 ppm of Se and as much as 73% of it was detected in the nuclear fraction [70]. Many studies revealed the lack of correlation between glutathione peroxidase activity and the total Se content in various animal fluids and organs [43], [32], [151], [144] indicating strongly the possibility of the existence of other selenoproteins.

The administration of radioisotope ^{75}Se to animals either *via* injection or in the diet was a widely used method in the search for new Se-binding proteins. Labelled proteins were then resolved and characterized using various biochemical separation techniques. Rat kidney cytosol was investigated by Motsenbocker and

Tappel [43]; three selenoproteins were resolved by gel filtration, two with MWs less than 40 kDa and one with MW of 75 kDa. Selenium in a 75-kDa protein was in the form of selenocysteine as determined by amino acid analysis.

Two-dimensional gel electrophoresis was applied to study Se-containing proteins synthesized by mouse mammary gland cells [40]. Eleven selenoproteins were detected and the five major ones had MWs of 14 kDa, 18 kDa, 22 kDa, 56 kDa and 58 kDa. For the purpose of that study, the term "selenoprotein" was used for proteins in which Se was still detected after a rigorous extraction and separation procedure.

Several rat tissues, namely brain, thyroid, testes, adrenals, heart, muscle and liver were studied by Behne *et al.* [151]. Thirteen Se-containing proteins or protein subunits with MWs ranging from 12.1 kDa to 75.4 kDa were detected in the tissue homogenates using SDS-PAGE. Most of these proteins were found in all investigated tissues but only one was detected in the thyroid (MW of 27.8 kDa) and one in the testes (MW of 19.7 kDa). The chemical forms of Se in these proteins were not investigated.

Selenium-containing proteins in the microsomal fractions of rat kidney and liver were studied by Viljoen *et al.* [41]. After solubilization with either SDS or Triton X-100 microsomes were subjected to the SDS-PAGE. In addition to glutathione peroxidase, seven ^{75}Se-labelled subunits with MWs from 17 kDa to 55 kDa were detected. Despite the treatment with SDS and 2-mercaptoethanol prior to the electrophoresis, ^{75}Se radioactivity was still detected in the protein bands.

This observation provided evidence for the authors that Se was covalently bonded to the proteins.

Jayawickreme [70] investigated selenoproteins in nuclear fraction of bovine kidneys. An extraction procedure involving the use of four different buffers was developed for this purpose. Ion exchange and gel filtration chromatography experiments were then applied to the protein extracts. Three proteins with MWs between 20 and 80 kDa, not observed previously, were reported and purified to a large extent.

Selenium in rat liver and its subcellular fractions were thoroughly investigated by Behne *et al.* [150]. Twelve Se-containing proteins with MWs between 12.1 kDa and 75.4 kDa were found. Some proteins were never reported before while others were already detected by the same or different research groups in various animal tissues. Among those previously reported, a protein with a MW of 18 kDa could be the phospholipid hydroperoxide glutathione peroxidase purified by Ursini *et al.* [152], [146]; a protein with MW of 19.7 kDa detected in testes could be the same as the one reported by Calvin [34]; a protein with MW of 27.8 kDa was observed previously in rat thyroid, liver and kidney by Behne *et al.* [151]; a protein with MW of 55.5 kDa could be a subunit of an 80-kDa protein detected previously in plasma by Burk and Gregory [153] and Motsenbocker and Tappel [154]. The possibility of the incorporation of Se into proteins by random replacement of S or by non-specific binding was ruled out by the authors [151] on the basis of the following two observations: (i) after SDS-PAGE no ^{75}Se was

detected in gel areas with high protein density and thus high S content; and (ii) after a test of *in vitro* incubation of the liver homogenate with [^{75}Se]selenite, no labelled protein was detected in the electrophoresis experiment.

Once it became evident that there are new Se-containing proteins present in animal organs, research started to focus on their further purification and characterization. That led to the discovery of several selenoproteins whose specific biological functions in living organisms have been established or at least postulated. Some of these proteins are described below.

Muscle Selenoprotein. This small protein of MW of 10 kDa was first observed by Pedersen *et al.* [155] in the supernatant fraction of heart and semitendinosus muscle of healthy lambs, but not in tissues affected by white muscle disease (WMD). It was then suggested that this protein might be involved in the prevention of Se-responsive myopathy in lambs. *In vitro* incubation of muscle homogenate with ^{75}Se did not reveal any binding of Se to proteins. Further purification was attempted by Whanger *et al.* [156] who reported the presence of a heme group as a part of the molecule. Beilstein *et al.* [33] provided evidence of Se being present as selenocysteine in this molecule. The procedure for purification of this protein was further developed by Vendeland *et al.* [157] using rat muscle. In addition to the previous findings, they determined that 1 g-atom of Se is present per molecule.

Sperm Selenoprotein. The impaired reproductive processes and the occurrence of nonmotile spermatozoa in case of Se deficiency was observed in

rats by Wu *et al.* [158]. Further investigations located Se in the midpiece region of the spermatozoon in the form of a polypeptide of MW of 17 kDa confined to the capsule that surrounds the sperm mitochondria [34]. Studies by Calvin et al. [159] confirmed that mitochondrial capsule protein (MCP) and Se-containing polypeptide are identical. The association of Se with this protein suggested a structural function for this trace element in the sperm although its chemical form has not yet been determined. Behne *et al.* [151] who detected a 19.7-kDa protein in rat testes suggested that this protein might be identical with the one purified from rat sperm.

Plasma Selenoprotein P (PSP). Binding of Se to plasma proteins in rats has been reported in 1973 by Burk [160]. Later experiments detected ^{75}Se in rat liver associated with a 83-kDa protein and in rat plasma with a 79-kDa protein [153]. It was named selenoprotein P. In case of Se deficiency, the incorporation of Se into this protein had a priority over glutathione peroxidase. This protein was further characterized by Motsenbocker and Tappel [161], [154], Motchnik and Tappel [162], and Read *et al.* [163] and purified using monoclonal antibodies by Yang *et al.* [36]. This is a glycoprotein of MW of 80 kDa. It consists of two subunits and Se is present in a 57 kDa polypeptide which after deglycosylation migrates at 43-kDa on SDS-PAGE. This is the first selenoprotein described that has multiple selenocysteine molecules and contains more than 1 g-atom of Se per polypeptide chain, *i.e.* 7 atoms per molecule. Isoelectric focusing under nondenaturing conditions gave a pI of 5.4.

Most functions of selenoprotein P are still unknown. It accounts for about

65% of the total Se in rat plasma [163]. Experiments using ^{75}Se-labelled PSP revealed that Se from this protein was first taken up in the body by brain [164] and testes [165] and within the latter it was transferred to the lysosomal and microsomal fractions. No enzymatic activity or biological function of this protein has yet been demonstrated. Some properties of this protein might suggest its role in Se transport. Firstly, it is a plasma protein that incorporates Se very rapidly after administration [153]; secondly, the appearance of Se in it precedes the incorporation of the element into tissues [154].

Type I Iodothyronine 5'-Deiodinase. A 27.8-kDa selenoprotein previously found in rat thyroid, kidney and liver by Behne *et al.* [151] was later shown to be present predominantly in liver mitochondria and microsomes [150]. In 1987, it was reported by Beckett *et al.* [166] that Se deficiency affected thyroid functions and the hepatic 5'-deiodination from L-thyroxine (T_4). It was suggested that the system responsible for the hepatic deiodination was a single microsomal enzyme. Investigations by Behne *et al.* [37] on comparison of the unknown selenoprotein and type I iodothyronine 5'-deiodinase (5'-D) identified them as the same enzyme. Later studies by Berry *et al.* [167] proved that Se in the 5'-D enzyme exists in the form of selenocysteine. This revealed one more important regulatory function of Se in animal physiology: its interaction with iodine which is another essential trace element.

Other Selenoproteins. Two Se-binding proteins from mouse liver cytosol were purified by Bansal *et al.* [168]. Their MWs determined using SDS-PAGE

were 14 kDa and 56 kDa. The amino acid sequence of a 14-kDa protein showed 92% sequence homology with rat liver fatty acid-binding protein [35]. Delipidation or SDS treatment did not remove Se from the protein but the nature of the Se binding is still unknown. For both proteins, possible involvement in Se anticarcinogenic properties has been proposed [169]. More recently, Pumford *et al.* [170] found a homology of a 56-kDa protein with a 55-kDa protein that covalently binds a metabolite of acetaminophen which is a hepatotoxic agent.

4.2 EXPERIMENTAL

This section describes various bioanalytical techniques used to purify and characterize selenoproteins. A complete description of the INAA method including sample preparation and a quality assurance program was presented in Chapter 2. Detailed data on Se concentration in the homogenate and subcellular fractions and on the preparation of cytosol were given in Chapter 3. Methods such as ion exchange chromatography, gel filtration, conventional and SDS-PAGE, and isoelectric focusing are presented here.

4.2.1 Chemicals and Reagents

All chemicals and reagents were purchased from the Sigma Chemicals, USA, unless otherwise noted. The presence of Se and other impurities in lithium hydroxide (BDH Chemicals, Toronto), HEPES and Ultrogel AcA 34 (Fisher

Scientific), used during gel filtration chromatography was evaluated using INAA. A sample of Ultrogel was equilibrated with a buffer as used in our experiments and then analyzed by INAA.

Elemental impurities present in sucrose were presented in Chapter 2; impurities in Tris, ammonium sulfate and DEAE-Sepharose are discussed in Chapter 5. Selenium was not detected in any of these chemicals.

4.2.2 Analytical and Bioanalytical Techniques

4.2.2.1 Fractionation by Precipitation with Ammonium Sulfate

Enough solid ammonium sulfate was added gradually to the cytosol fraction to make a concentration of 1 M while slowly stirring on the ice bath. The mixture was centrifuged at 5 000xg for 15 min and the pellet was discarded. Further additions of ammonium sulfate were then made to the supernatant for a final concentration of 2 M. Formulas for calculating the amounts of salt required were taken from Scopes [171]. The precipitated protein was collected by centrifugation and the supernatant was discarded. The pelleted protein was redissolved in a minimum volume of a 0.1 M Tris/Ac buffer (pH 8.3).

4.2.2.2 Ion Exchange Chromatography

The experiments were performed at 4°C. A slurry of DEAE-Sepharose gel (Pharmacia) was packed into a 3x30 cm column and the column was equilibrated with 3 bed volumes of 0.1 M Tris/Ac buffer (pH 8.3). A volume of 100 mL of

cytosol was concentrated 1.5-fold using Aquacide (CALBIOCHEM) and then dialysed against Tris buffer before it was applied to the column. A flow rate of 0.75 mL min^{-1} was selected and fractions of 6 mL were collected. One bed volume of buffer was passed through the column to wash out the non-bound material and then a linear gradient of sodium chloride in Tris buffer of up to 1 M salt was added to elute the bound proteins. Fractions with optical density at 280 nm were dialysed against water, transferred into medium-sized irradiation vials and analyzed for Se by INAA.

4.2.2.3 Gel Filtration Chromatography - Column Calibration and Sample Run

A column of 3x100 cm packed with Ultrogel AcA 34 (Pharmacia) was used in this study. Experiments were performed at 4°C. The column was calibrated with selected standards from the Sigma MW-GF-200 Molecular Weight Kit. The standards were: β-Amylase 200 kDa, Alcohol Dehydrogenase 150 kDa, Carbonic Anhydrase 29 kDa and Cytochrome c 12 kDa. Blue Dextran 2 000 kDa was used to determine column void volume (V_o). A flow rate of less than 0.7 mL min^{-1} and a fraction volume of 3.5 mL were selected. This flow rate was calculated from recommendations of the manufacturer of the gel [172]. Protein elution was monitored by measuring absorbance at 280 nm. A sample of 0.1 M HEPES buffer (pH 7.4) was used throughout all experiments. Sodium azide 0.05% was added to the buffer to prevent microbial growth in the column during the periods the column was not in use.

Two methods of sample concentration prior to the gel filtration experiments were employed. These were either concentration using Aquacide or precipitation of proteins with ammonium sulfate. The samples were clarified by centrifugation before applying them to the column. If the experiment was to be followed by INAA, fractions were collected directly in medium-sized irradiation vials, then dried under an IR lamp and analyzed for Se.

4.2.2.4 Analytical Polyacrylamide Gel Electrophoresis

4.2.2.4.1 Apparatus

Polyacrylamide gel electrophoresis (PAGE) was carried out using a LKB 2050 Midget Electrophoresis Unit connected to a LKB 2197 Model power supply and a Haake Model F3-C multi-temperature thermostatic circulator. Experiments were run at 6°C with a constant voltage mode at 200 V and were terminated after the band of tracking dye reached the bottom of the gel.

4.2.2.4.2 Preparation of Reagents and Gels

Reagents and gels were prepared according to the LKB 2050 Midget Electrophoresis Unit Lab Manual, LKB 2117 Multiphor Application Note 306 and on the basis of other procedures recommended by Hames [173]. They were checked for Se contamination using INAA. Three gel concentrations, *viz.* 7.5%, 10% and/or 12.5% were used in the experiments.

4.2.2.4.3 PAGE in Non-Denaturing Buffer System

Gel electrophoresis of native proteins under non-dissociating conditions is designed to fractionate a protein mixture in such a way that subunit interaction, native protein conformation and biological activity are preserved. Separation of native proteins occurs on the basis of size, shape and charge. In the initial phase of studies on selenoproteins, experiments were done in order to find out the number of proteins present in the samples at the end of the purification procedure. An attempt to determine the molecular weights of the native proteins was also made.

4.2.2.4.3.1 Calibration Curve

The procedure for the determination of molecular weights in a non-denaturing system is described in the Sigma technical Bulletin No. MKR-137 [174]; it is a modification of the methods of Bryan [175] and Davis [176]. Briefly, standard proteins were electrophoresed on polyacrylamide gels at three different concentrations, *viz.* 7.5%, 10.0% and 12.5%. The mobility of a protein in each gel relative to the mobility of the tracking dye (R_f) was determined and $100\log(R_f 100)$ was plotted against the percent gel concentration for each protein (known as Ferguson plots). The logarithm of the negative slope was plotted against the logarithm of the molecular weight of each protein. A linear plot was produced from which the molecular weight of an unknown protein could be estimated.

Protein markers from the Sigma MW-ND-500 Molecular Weight Kit were

used for this purpose. The standards included: Urease 272 kDa (trimer), Bovine Serum Albumin 132 kDa (dimer), Chicken Egg Albumin 45 kDa (monomer) and Carbonic Anhydrase from bovine erythrocytes 29 kDa. Protein standards were handled according to the recommendations in the bulletin accompanying the protein kit [174]. Fifty µL of diluted protein was mixed with 50 µL of sample buffer and applied to the sample well in the gel plate. Samples were run in duplicates.

4.2.2.4.4 SDS - PAGE

Most studies on proteins using polyacrylamide gel electrophoresis (PAGE) apply a buffer system that would dissociate all proteins into their polypeptide subunits. The most common dissociating agent used is the ionic detergent sodium dodecyl sulfate (SDS). Under denaturing conditions and in the presence of a disulfide cleaving reagent (*e.g.*, 2-mercaptoethanol, dithiothreitol, *etc.*), most polypeptides bind SDS in a constant weight ratio. Consequently, they possess a negative charge of equal value per unit weight provided by the bound detergent and therefore they migrate in polyacrylamide gels according to polypeptide size. The distances migrated by standard proteins are used to prepare a calibration curve from which the molecular weight of the unknown can be determined.

4.2.2.4.4.1 Calibration Curve

Six protein markers from the Sigma MW-SDS-200 Molecular Weight Kit were used to construct a calibration curve. These were: Carbonic Anhydrase 29

kDa, Albumin from Egg White 45 kDa, Bovine Serum Albumin 66 kDa, Phosphorylase B 97.4 kDa (subunit), β-Galactosidase 116 kDa (subunit), and Myosin 205 kDa (subunit). For the purpose of constructing a calibration curve, relative mobility (R_f) of each standard was calculated by dividing its migration distance from the top of the gel to the centre of the protein band by the migration distance of the Bromophenol Blue tracking dye from the top of the gel. The R_f values (abscissa) were plotted against the known molecular weights (ordinate) on a semi-logarithmic paper.

4.2.2.5 Isoelectric Focusing

4.2.2.5.1 Apparatus

Isoelectric focusing (IEF) experiments were carried out using a LKB 2050 Midget Electrophoresis Unit connected to a LKB 2197 Model power supply and a Haake Model F3-C multi-temperature thermostatic circulator. Experiments were run at 5°C with a constant power mode at 3 W and they were terminated after about 3.5 h. The pH gradient along the gel was determined using either a LKB Model 2117-111 surface electrode or using marker proteins of known pI values.

4.2.2.5.2 Preparation of Reagents and Gels

Reagents and gels were prepared using the LKB 2117 Multiphor Application Note 250 and the LKB 1804 Instruction Manual for LKB Ampholine PAGplates and they were checked for Se contamination using INAA. The cathode solution was

0.1 M NaOH while 0.1 M H_3PO_4 was used as the anode solution. A 5% acrylamide gel containing 2% carrier ampholytes (Ampholine LKB) with pH range 3.5 - 10.5 was casted in preliminary IEF experiments and the same gel concentration with 2% carrier ampholytes (Ampholine, preblended) of pH 4.0 - 6.5 was used in later studies. Gels were 1.50 mm thick. A 0.1% Triton X-100 solution was included in the gel. Samples were dialysed against water for 2-3 h and applied to the top of a gel (cathode end) in 2% ampholyte solution.

4.2.2.5.3 Calibration Curve

Five protein markers with known pI values from the Sigma IEF-M2 Kit were used to construct a calibration curve. These were: Glucose Oxidase from *Aspergillus niger*, pI 4.2; Trypsin Inhibitor from soy bean, pI 4.6; β-Lactoglobulin from bovine milk, pI 5.1; Carbonic Anhydrase II, from bovine erythrocytes, pI 5.4; and Carbonic Anhydrase II, from bovine erythrocytes, pI 5.9. Samples were prepared according to the information provided in the bulletin accompanying the protein kit.

4.2.3 Precipitation of Hemoglobin

Hemoglobin was precipitated from cytosol according to a slightly modified procedure of that described by Yoshida and Watanabe [177]. Briefly, ethanol-chloroform (2:1, v/v) cooled to -20°C was added dropwise to a cytosol sample (1:3, v/v). After stirring for 20 min in an ice bath, the hemoglobin precipitate was

collected by centrifugation in glass tubes (15 000xg, 20 min). Ethanol, cooled to -20°C was then added dropwise to the supernatant (2.5:1, v/v). The mixture was stirred for 20 min in an ice bath and centrifuged in a precooled rotor at 15 000xg for 15 min. The precipitate was dissolved in 0.1 M Tris buffer (pH 8.3) and a sample applied to a DEAE-Sepharose column.

The loss of Se during this experiment was investigated. Smaller cytosol samples of 20 mL each were used for this purpose. All consecutive fractions of the experiment were dried under an IR lamp and analyzed for Se content using INAA.

4.2.4 Determination of Protein Content

Three different methods were used to determine protein concentration. These were: (i) the Lowry method [94], described in Chapter 3, Section 3.2.5; (ii) the Bio-Rad Protein Assay based on the method of Bradford [178]; and (iii) the BSA Protein Assay (Pierce Chemical Co.) based on the method of Smith *et al.* [179] and Wiechelman *et al.* [180]. Bio-Rad's disposable polystyrene cuvettes were used in the second procedure. A solution of crystalline bovine serum albumin was applied as a protein standard in all cases.

4.3 RESULTS AND DISCUSSION

4.3.1 Contaminants Present in Chemicals and Reagents

No Se was detected in any reagents and chemicals used in the electrophoresis and isoelectric focusing experiments.

Elemental contaminants detected in lithium hydroxide, HEPES and Ultrogel AcA 34 are presented in Table 4.1. No Se was present in these reagents. Besides elements such as Br, Cl, K and Na which are typically present in trace amounts in majority of the chemicals analyzed, several other elements were detected. Iodine was found in HEPES but at an insignificant level of 0.04 ppm to be a potential contaminant. Magnesium was detected in the gel (2.48 ppm) but its concentration was ten times smaller than that in cytosol (21.5 ppm, Table 5.1). The most contaminated reagent was lithium hydroxide. In addition to traces of Br, Cl, K and Na, it contained high levels of V (0.35 ppm) - about 50 times higher than that in cytosol, and Zn (1.84 ppm) - approximately the same order of magnitude as in cytosol. Lithium hydroxide was used in very small quantities only to adjust the pH of the HEPES buffer. In case of the investigations of Zn and V, however, chemical of better quality than the one used in this studies should be employed to minimize the possibility of contamination.

Table 4.1

Contaminants detected in lithium hydroxide, HEPES and Ultrogel AcA 34[a]

Element	Concentration (ppm)		
	Lithium Hydroxide	HEPES	Ultrogel AcA 34
Br	0.151 ± 0.001	0.26 ± 0.03	0.191 ± 0.004
Cl	3.79 ± 0.92	3.58 ± 0.44	7.89 ± 0.01
I	< 0.02	0.04 ± 0.01	< 0.02
K	10.88 ± 0.27	2.59 ± 0.14	< 0.36
Mg	< 1.25	< 1.44	2.48 ± 0.34
Na	39.3 ± 2.5	1.04 ± 0.04	10.8 ± 0.3
V	0.35 ± 0.02	< 0.004	< 0.006
Zn	1.84 ± 0.23	< 0.20	< 0.20

[a] average of 6 determinations.

4.3.2 Calibration Curves

4.3.2.1 Gel Filtration Chromatography

A calibration curve obtained using molecular weight markers from the MW-GF-200 Kit and a 3x100 cm column of Ultrogel AcA 34 is presented in Fig. 4.2. Four standards were used to construct the curve. The column calibration regression equation was y = 7.1763 - 1.0828x and r = 0.9935.

4.3.2.2 PAGE in Non-Denaturing Buffer System

Two plots constructed for the determination of the molecular weight of an unknown protein in a PAGE experiment are presented in Figs.4.3 and 4.4, respectively. The calibration regression equations were:

in Fig 4.3:

Chicken Egg Albumin: y = 237.0 - 5.66x; r = 0.9822;

BSA (Monomer): y = 237.6 - 7.05x; r = 0.9836;

BSA (Dimer): y = 242.8 - 10.65x; r = 0.9980;

Urease (Trimer): y = 240.5 - 14.03x; r = 0.9967;

and in Fig. 4.4:

y = -1.6155 + 0.5111x, and r = 0.9948.

4.3.2.3 SDS-PAGE

An example of a typical calibration curve obtained in the SDS-PAGE experiments where a 10% gel was used is shown in Fig.4.5. The gel calibration

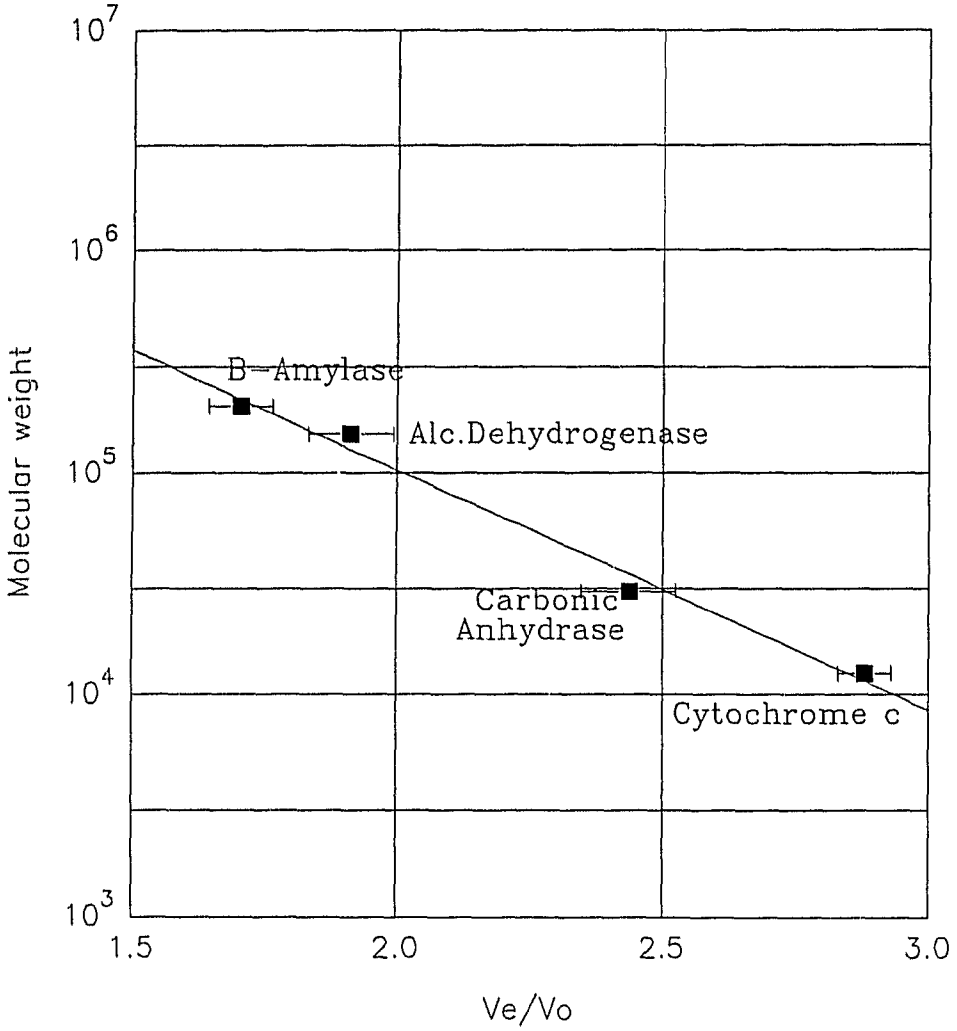


Fig. 4.2. Calibration curve for gel filtration chromatography.

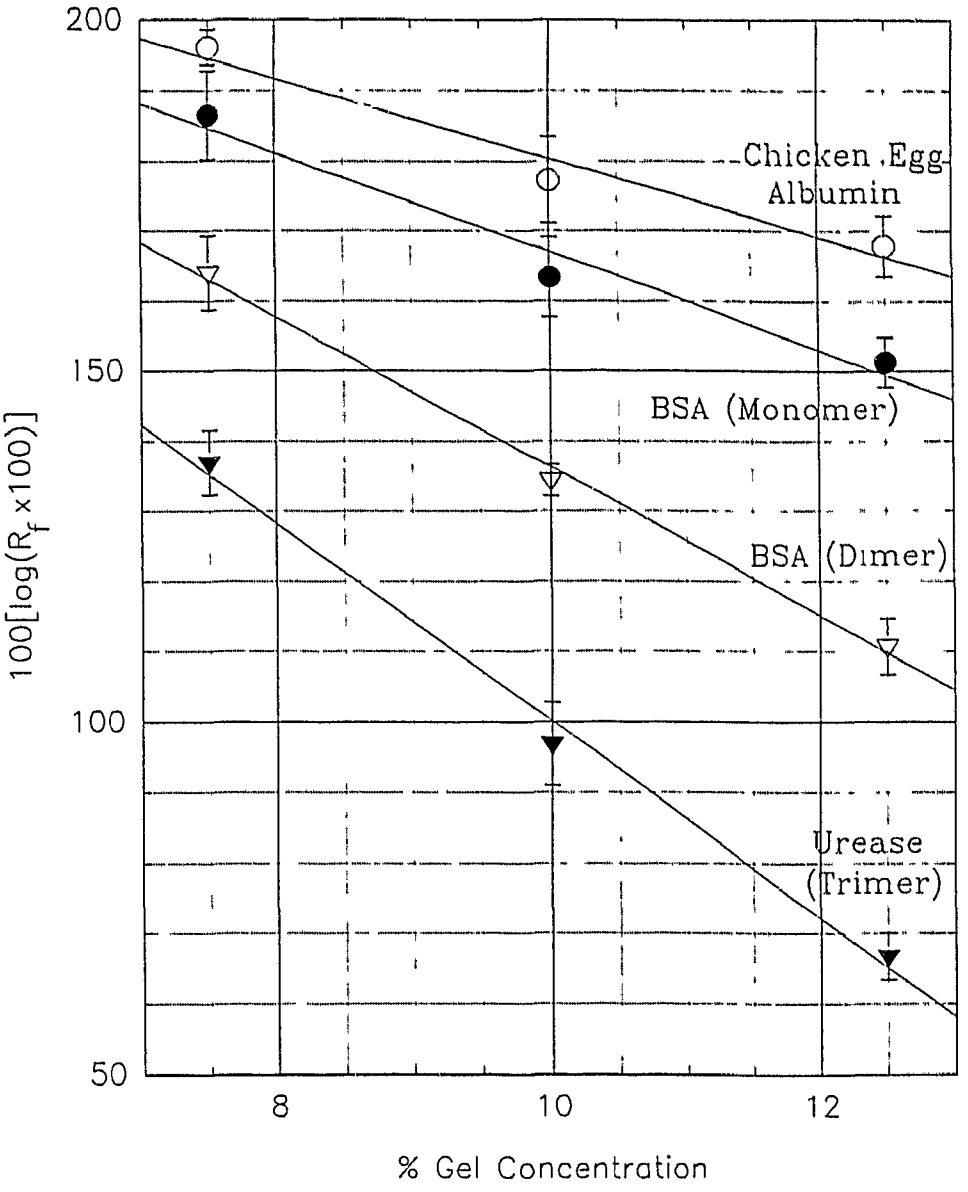


Fig. 4.3. Calibration curve for PAGE in non-denaturing buffer system.

Chicken Egg Albumin	(○)
BSA (monomer)	(●)
BSA (dimer)	(▽)
Urease (trimer)	(▼)

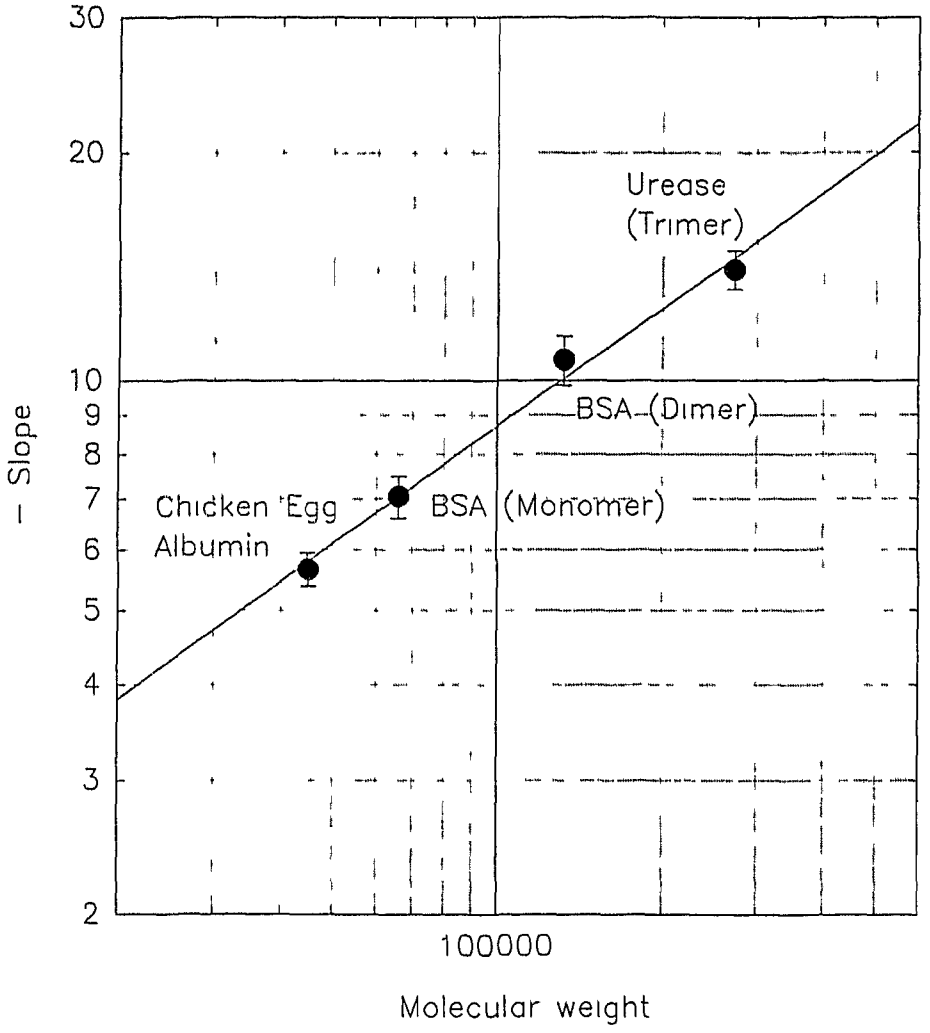


Fig. 4.4 A plot of the slopes determined from Fig. 4.3 *vs* molecular weight for the determination of molecular weights in non-denaturing PAGE.

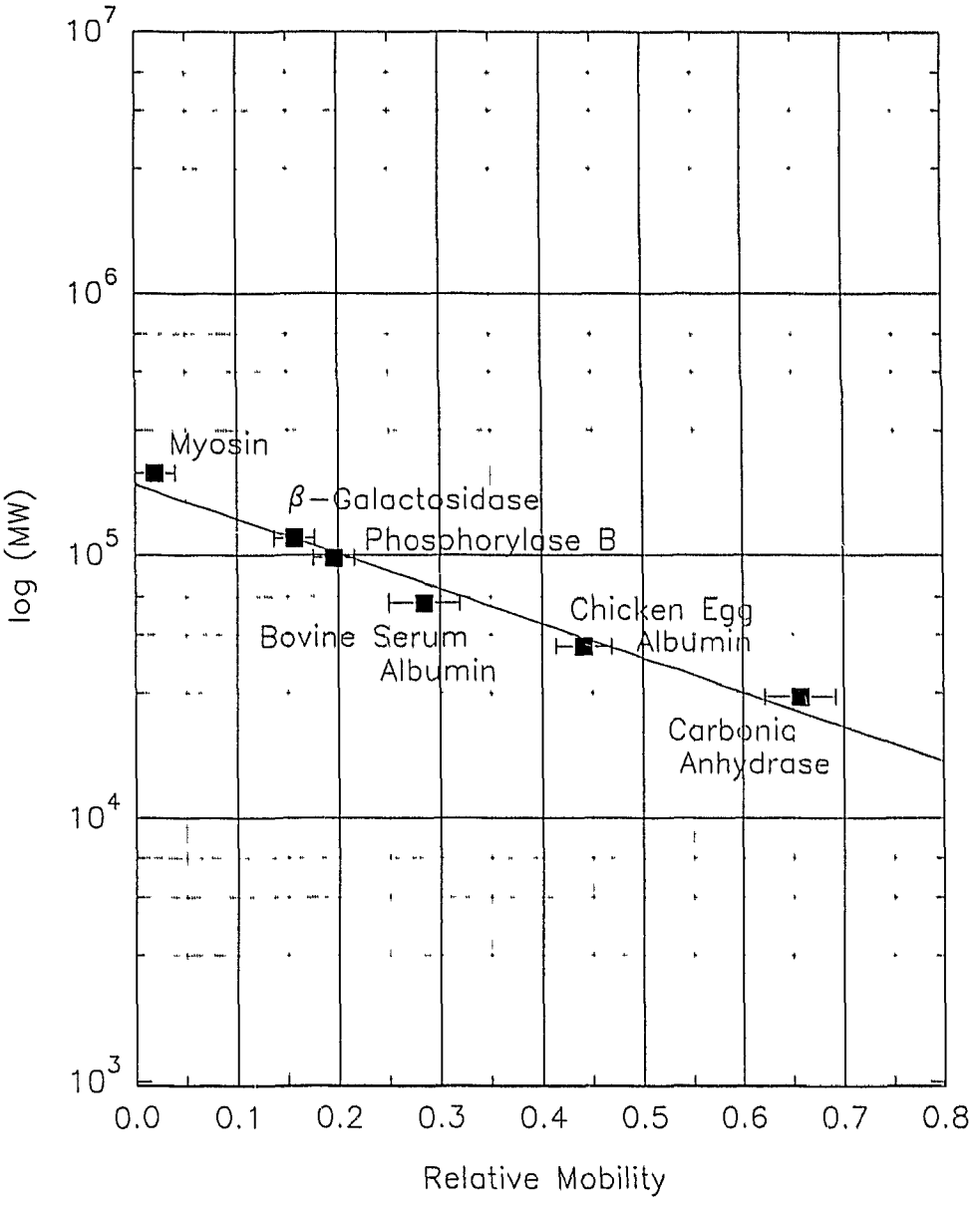


Fig. 4.5. Calibration curve for the determination of molecular weights in SDS-PAGE.

regression equation was y = 5.2686 - 1.3173x and r = 0.9831.

4.3.2.4 Isoelectric Focusing

An example of a calibration curve, obtained typically in IEF experiments is shown in Fig. 4.6. The gel calibration regression equation was y = 7.2455 - 0.0683x and r = 0.9944.

4.4 STUDIES ON SELENOPROTEINS IN THE CYTOSOL FRACTION

Results on the total Se content of bovine kidney and its subcellular fractions were presented in Chapter 3. As discussed earlier, Se in cytosol accounted for only 3.7 - 10.3% of the Se present in kidneys (Table 3.3). The variation is most likely due to the variation of Se content in the kidney itself. The purity of cytosol with respect to other fractions was determined using several marker enzymes and the data were presented in Tables 3.8 through 3.12. On the basis of the results obtained it was decided to proceed with investigations on separations without further purification of cytosol.

4.4.1 Preliminary Studies

The information available in the literature on the nature and behavior of selenoproteins in bovine kidney cytosol is rather limited. A study was done in our laboratory by Froom [181] who noticed that ammonium sulfate precipitation caused

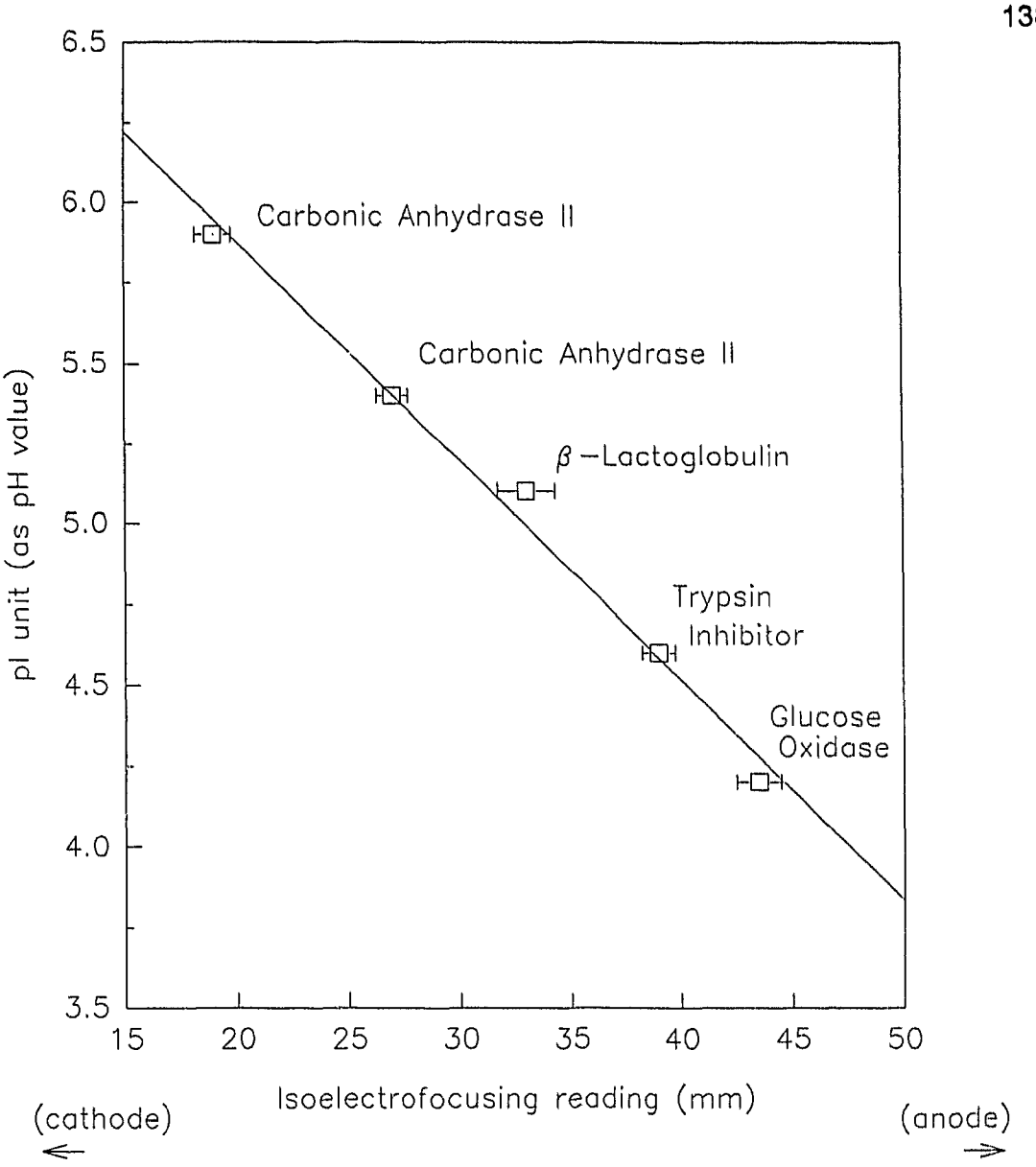


Fig. 4.6. Calibration curve for the determination of pI in Isoelectric Focusing.

protein denaturation when applied to cytosol. This observation was used in the initial stages of the present work. No introductory purification step was done to the crude cytosol prior to chromatography. The selections of appropriate pH values for anion exchange and gel permeation chromatography experiments were also based on Froom's results. He examined the effect of pH on the extractability of Se in the supernatant fraction and selected a pH of 8.3 for the ion exchange chromatography and a pH of 7.4 for gel filtration. When necessary, samples were concentrated using Aquacide, an agent used by Jayawickreme [70] in this laboratory.

In the beginning, the possibility of applying gel filtration chromatography to the crude cytosol was examined. A sample of 40 mL of cytosol was concentrated with Aquacide to 5.5 mL and then dialysed against 0.1 M HEPES buffer (pH 7.4) to give a final volume of 8 mL. This degree of concentration gave a final protein concentration of about 40 mg mL^{-1} or about 300 mg of protein in total. It was a matter of compromise to select the proper sample size. Too much protein loaded on a column would negatively influence the resolution; too concentrated a sample passing between the gel beads could block the solvent flow through the column due to high viscosity. On the other hand, too small a sample would not generate enough material in the effluent for further characterization by INAA. An example of a chromatography experiment is shown in Fig. 4.7. As anticipated *a priori*, a very poor separation was observed due to the large numbers of proteins in the sample analyzed. Stellwagen stated that no more than 10 proteins could be

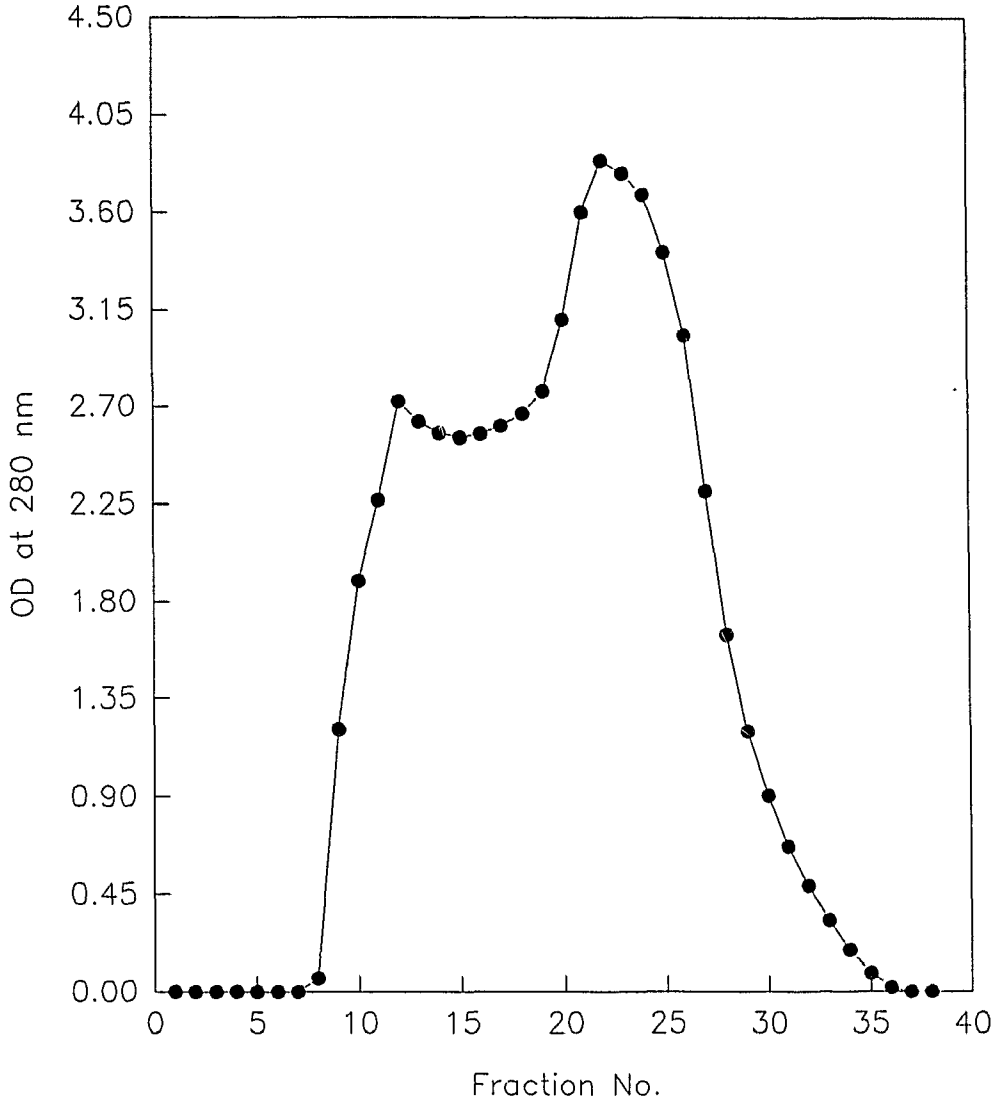


Fig. 4.7. Gel filtration chromatography of cytosol on Ultrogel AcA 34.

resolved from one another on a gel filtration column [182]. Only if the protein of interest has a molecular weight considerably different from the bulk of other macromolecules present, can this technique provide a significant enhancement in purification. In our case, the nature of selenoprotein(s) was unknown, therefore the highest purification factor would be desirable. Moreover, due to the high dilution factor in the effluent the application of INAA to determine Se in the collected fractions was not possible. At this point, it was decided to perform gel filtration chromatography later in the purification procedure when the number of other proteins in a test sample would be small. We also thought that the degree of resolution would increase if a protein mixture was fractionated in an earlier step on the basis of a completely different property.

In the next step, cytosol was subjected to ion exchange chromatography on a DEAE-Sepharose column. Protein (solid line) and Se (dashed line) elution profiles are presented in Fig.4.8. It is evident that the proteins separated into three distinct peaks: the first one representing material that did not bind to the column, the second peak eluted at about 0.23 M NaCl and the third peak at about 0.36 M NaCl. The second and the third peaks contained other minor protein fractions that did not resolve well on the column. For the determination of Se, 3 or 4 protein-containing fractions were combined, dialysed and dried under an IR lamp before analysis. The INAA results indicate that at least five Se-containing proteins were present in cytosol. The peak that eluted at about 0.23 M NaCl had the highest Se concentration (up to 26 ppm). This Se peak always preceded the largest protein

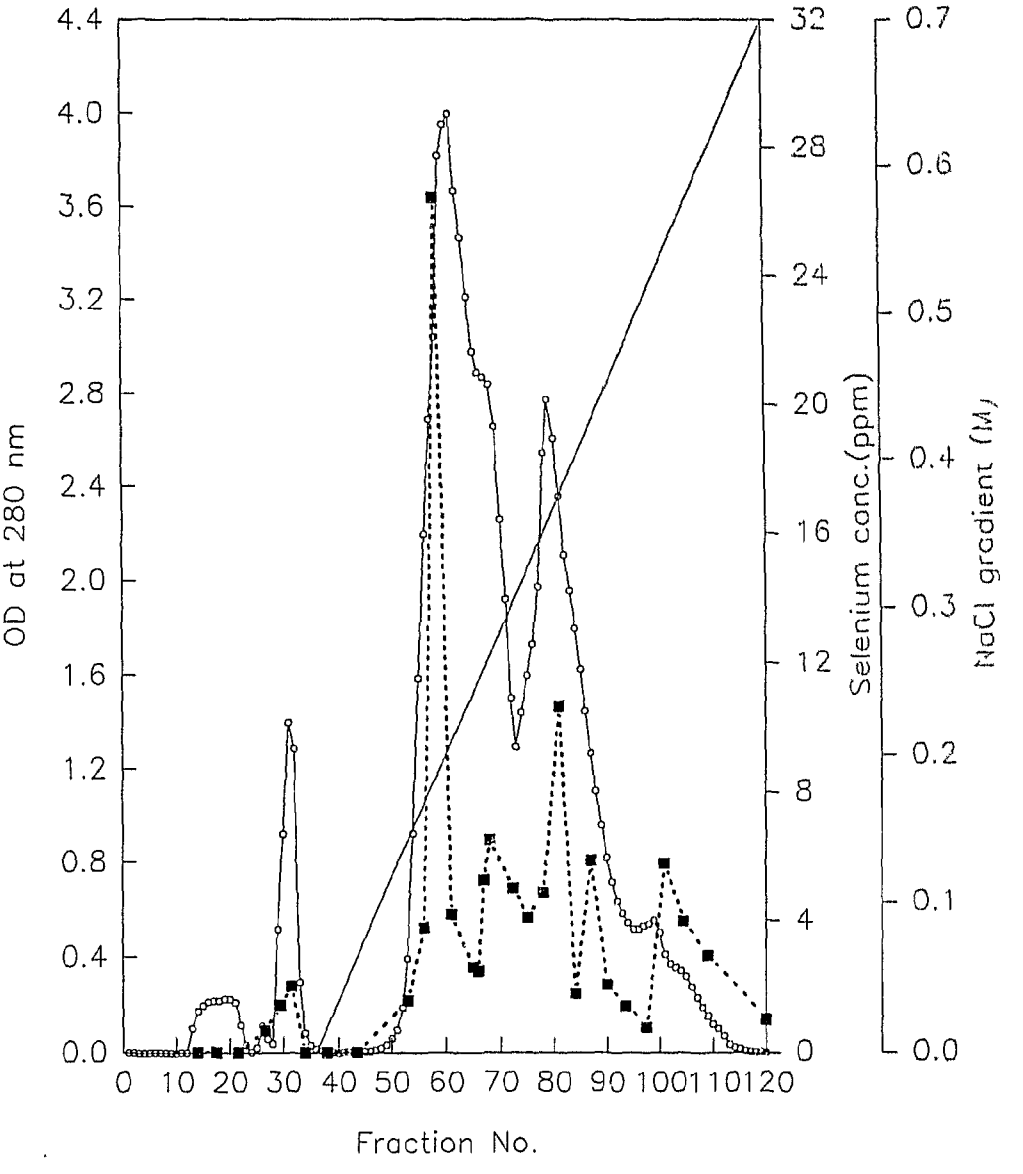


Fig. 4.8. Elution profiles of Se and protein in cytosol on a DEAE-Sepharose column.

protein (—o—o—o—o—)

Se concentration (ppm) (– –■– –■– –■– –)

peak eluted from the column, as observed in at least 10 experiments. Our initial efforts were focused on purification of this Se-protein.

In a different DEAE-Sepharose experiment, the material bound to the column was eluted stepwise with two bed volumes of 0.3 M NaCl. A salt concentration of 0.3 M was chosen in order to separate selenoprotein of interest from other selenoproteins present in cytosol and also to remove other, unwanted polypeptides, RNA, DNA, *etc.*, that would remain bound to the gel. Fractions corresponding to the eluted peak were pooled, concentrated with Aquacide to 7 mL, dialysed against 0.1 M HEPES buffer (pH 8.3), centrifuged and applied to a gel filtration column. Protein and Se elution profiles are presented in Fig. 4.9. This time, a better degree of protein separation was achieved compared to the previous gel filtration experiment (Fig 4.7). Two major peaks were observed. Most of the protein (as monitored by OD at 280 nm) eluted in the column void volume. The second peak with a maximum at fraction No. 35 consisted probably of two or three components as judged from the shape of the peak. The number of proteins that eluted in the column void volume was unknown at that stage, but the peak contained most probably several proteins with molecular weights exceeding the gel's exclusion limit, *i.e.* 750 kDa. Measurements of the absorbance at 260 nm did not reveal the presence of nucleic acids. Due to a very low initial volume (7 mL) of the sample loaded on to the column, Se could only be determined in the effluent after combining two or three fractions. Two Se peaks were found, the higher being eluted in the void volume. The other Se peak was quite broad and the MW of the

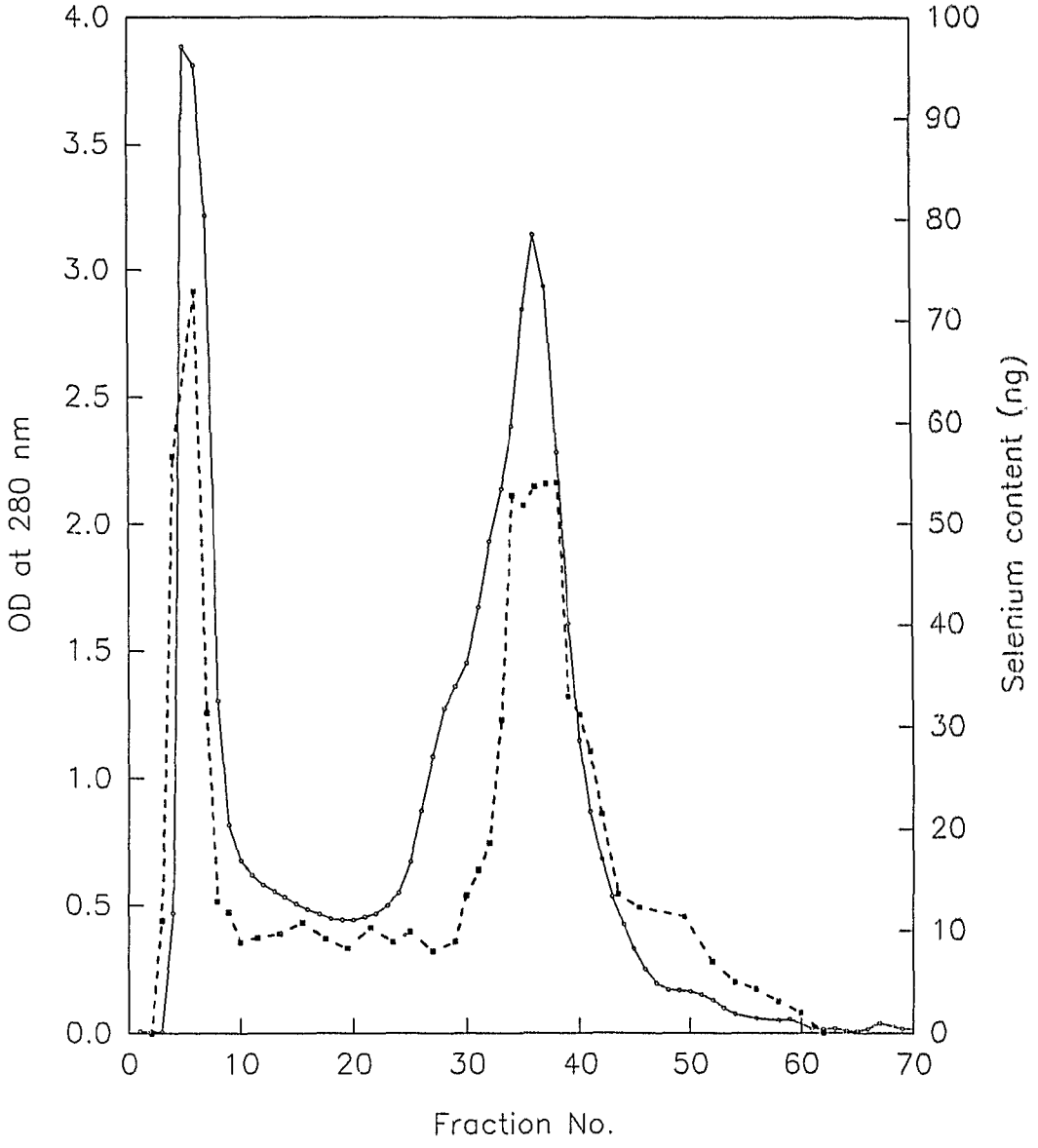


Fig. 4.9. Elution profiles of Se and protein on an Ultrogel AcA 34 column.

protein (—○—○—○—)

Se content (ng) (- -■- -■- -■- -)

associated protein was estimated from two different gel filtration experiments as 69 kDa and 72 kDa (assuming the top of a peak as fraction No.29 in Fig. 4.9). It was noticed that the Se peak overlapped strongly with another protein whose MW was estimated from two experiments as 39.5 kDa and 45.0 kDa.

In order to investigate the number of components in each peak, the Se-containing fractions were combined into two samples corresponding to the two major peaks eluted from the gel column, and the pooled fractions were subjected to PAGE in non-denaturing buffer system. Three different gel concentrations were used, *viz.* 7.5%, 10% and 12.5%. It was important to select the proper pH for this type of PAGE as proteins were subject to separation on the basis of both size and charge. The pH value had to be chosen within the range over which the proteins of interest are stable. Jayawickreme [70] studied the stability of Se-protein complexes in the supernatant fraction of bovine kidneys. He found that these complexes were quite stable and started to degrade only above pH 10. These observations left us with several pH values to select from. Another desirable information, namely the pI values of the proteins of interest, was not available at that time. Since the isoelectric points of the majority of known proteins lie between pH 4 and 7, the following pH values were selected: (i) pH of 6.8 for the stacking gel buffer; (ii) pH of 6.8 for the sample buffer; (iii) pH of 8.3 for the resolving gel buffer; and (iv) pH of 8.3 for the electrode buffer. Samples were dialysed overnight against sample buffer (without glycerol and Bromophenol Blue), centrifuged and diluted 1:1 with sample buffer just before electrophoresis.

Approximately 15 µL of such solutions were applied to the gel.

The peak that eluted in the void volume of the gel filtration column was expected to contain a mixture of large-size proteins and therefore to give several blue bands in the gel slab. However, a heavy stained band at the gel origin was observed in all gel concentrations used. This behavior of the "void volume" proteins was also observed in all later PAGE experiments, even in those which included SDS in the environment. It was concluded that this sample contained aggregated, denaturated proteins and that this denaturation was irreversible since the presence of SDS did not show any difference. This denaturation occurred most probably prior to electrophoresis either on a column or even during the sample concentration step.

The second peak showed four distinct bands with various intensities after staining. An attempt was made to determine the molecular weights of these proteins. Ferguson plots for all four unknown proteins are shown in Fig.4.10. Their negative slopes along with the molecular weights determined from the calibration curve of the protein standards are presented in Table 4.2. Calculated molecular weights were: 105.6 kDa for Protein I, 65.6 kDa for Protein II, 103.5 kDa for Protein III and 231.1 kDa for Protein IV. These results do not correlate well with those obtained using gel filtration. Closer examination of Fig. 4.10 might give some explanations of these artifacts. By using Ferguson plots, the charge factor is eliminated from all further considerations. This means that the retardation of a protein when it moves through gels of various pore sizes (as determined from the

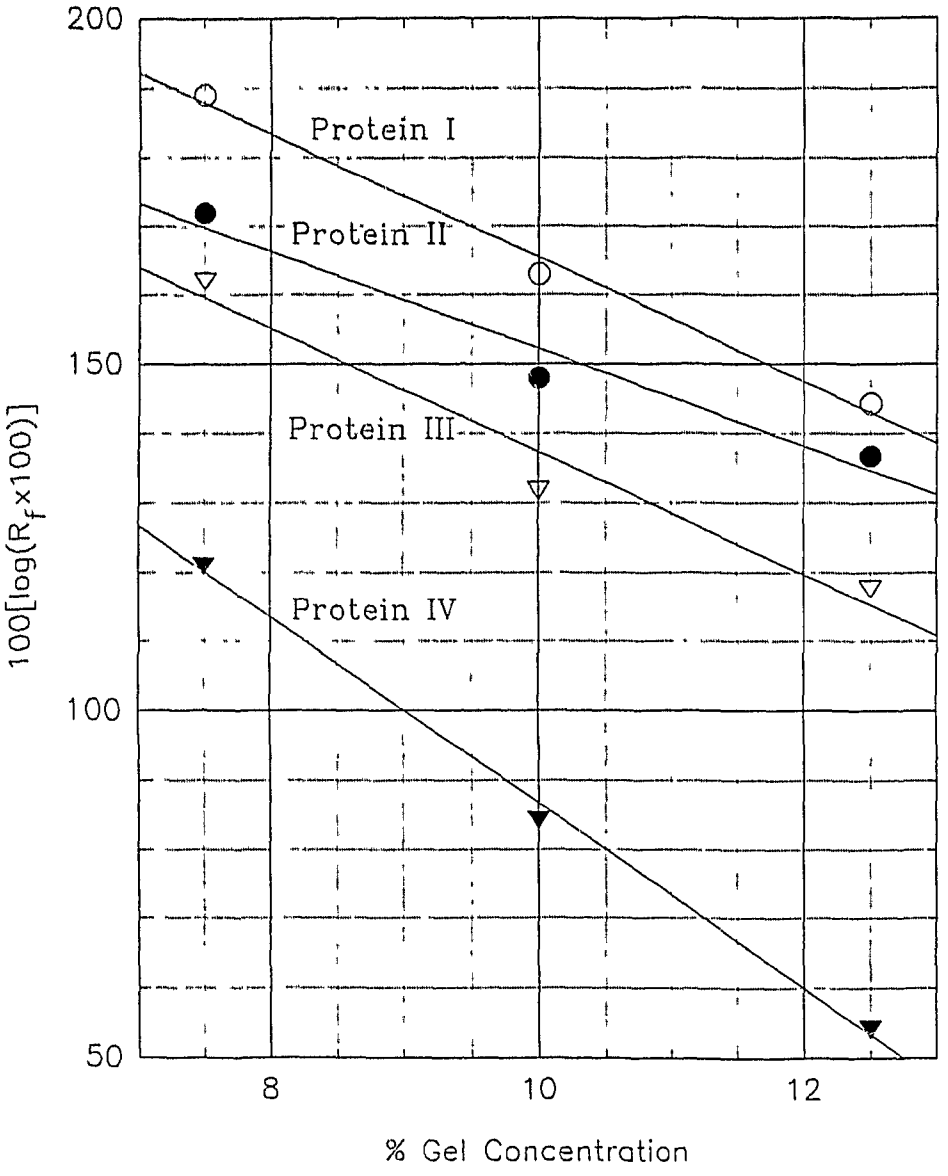


Fig. 4.10. Ferguson plots of four proteins resolved in non-denaturing PAGE.

Protein I	(○)
Protein II	(●)
Protein III	(▽)
Protein IV	(▼)

slope) depends only upon the size of a molecule or rather its Stokes radius. In the ideal situation, if all proteins were spherical globulins, all four line should come to one point at or near %T = 0. A modification of Fig. 4.10 where the abscissa is extended to the values of around 0% gel concentration is shown in Fig.4.11. It can be seen that the behavior of Protein I is unusual. This protein is most probably a very asymmetric molecule that contains a non-peptide moiety such as a glyco-, sulfo-, phospho-, or lypo-group. This type of behavior might also be due to a very large charge on a protein for which the Ferguson analysis could not account. As all MWs calculated from the electrophoresis experiment are much higher then the MWs estimated from gel filtration chromatography, all four proteins might have an asymmetric shape or be highly charged.

Table 4.2

Slopes and calculated molecular weights of proteins resolved in PAGE experiment in non-denaturing buffer system

Protein	Negative Slope	Molecular Weight (kDa)
Protein I	8.958	105.6
Protein II	7.022	65.5
Protein III	8.864	103.5
Protein IV	13.362	231.1

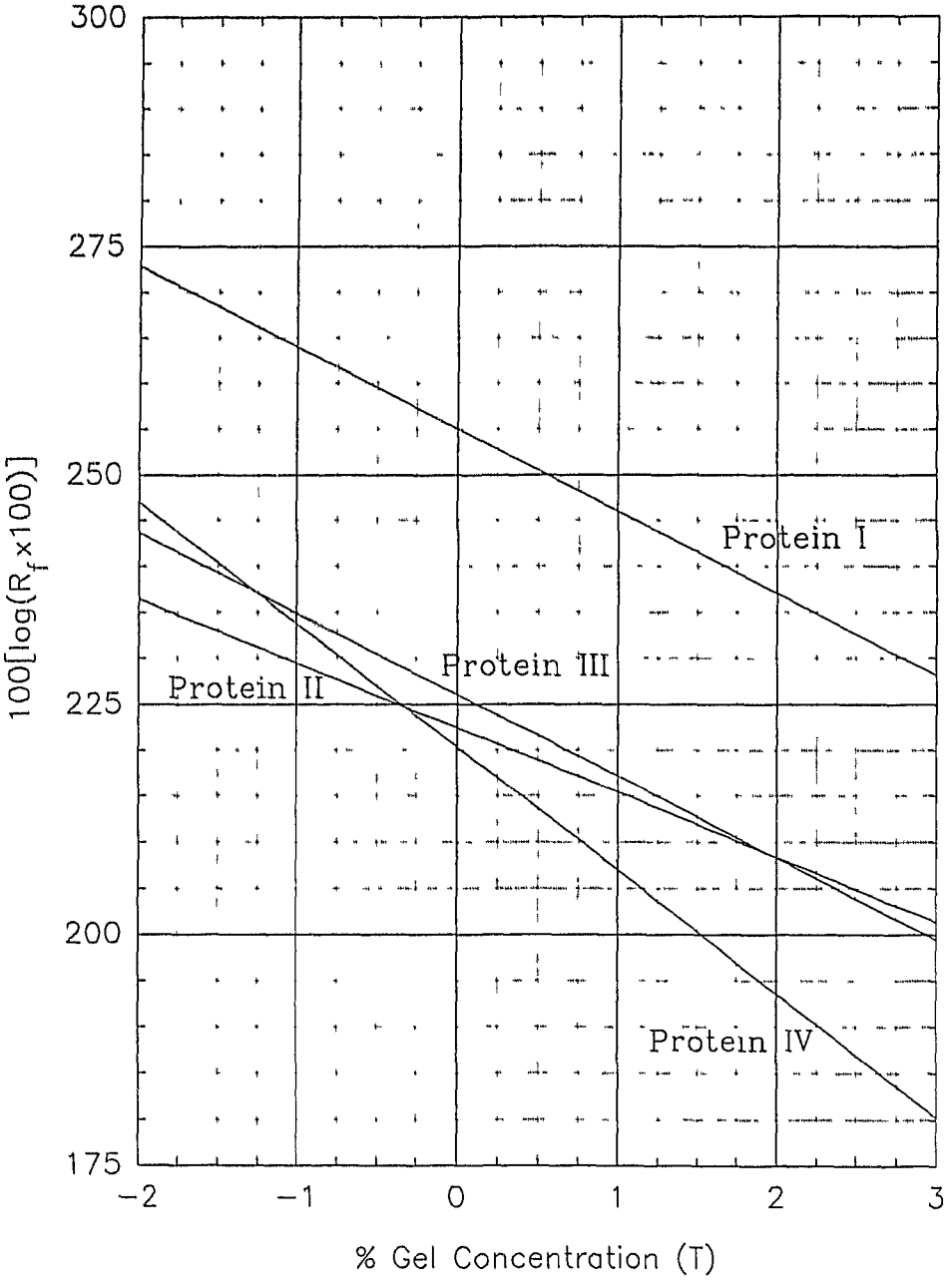


Fig. 4.11. Relative mobility of unknown proteins *vs* gel concentration around %T = 0.

4.4.1.1 Summary of Preliminary Experiments

From the preliminary investigations on Se-containing proteins in the cytosol fraction it was concluded that gel filtration chromatography cannot be effectively applied to crude cytosol because of too low resolution of this technique for such a complex sample. Chromatography on a DEAE-Sepharose column in conjunction with INAA showed Se in cytosol to be present in at least five distinct peaks. The largest Se peak from ion exchange chromatography was further purified using gel filtration. It separated it into two peaks, first one with MW > 750 kDa and the other one with an estimated MW of 69 -72 kDa. Non-denaturing PAGE applied to characterize these two peaks revealed the possibility of aggregation and denaturation of proteins that were eluted in the first peak from the column. The second peak separated on a gel into four proteins. An attempt to determine their MWs was unsuccessful. Proteins did not behave ideally in the Ferguson plot which may testify to the presence of high charge or asymmetric shape of the molecules.

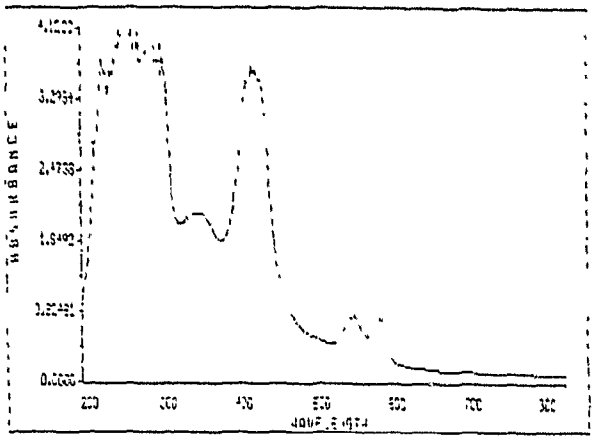
4.4.2 Purification of Selenoprotein A1

It was observed in both the ion exchange and gel filtration chromatography experiments that the major Se peak coeluted from the columns along with other proteins with high absorbance at 280 nm. Although cytosol (obtained by centrifugation at 110 000xg for 110 min) and supernatant (obtained by centrifugation at 30 000xg for 25 min) fractions were previously investigated in our

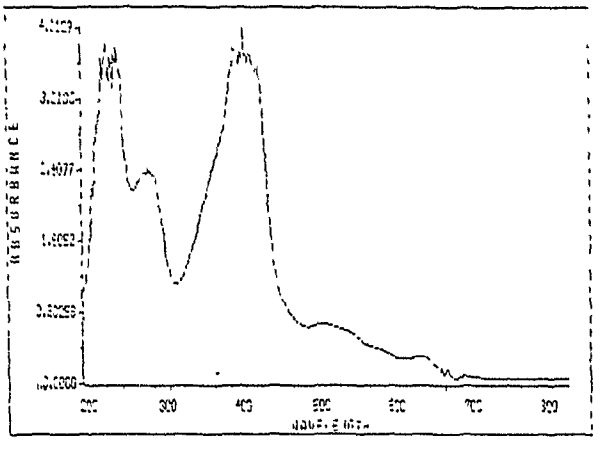
laboratory, none of the researchers reported on this interfering protein. The protein had a red color and its estimated MW was 39.5 - 45.0 kDa. We suspected that this protein might be hemoglobin or some products derived from it, judging by its characteristic red color. The other possibility could be that it was cytochrome c or an aggregate of it but as this protein is attached to mitochondria [183] its presence in the cytosol would be less likely. The MW of the protein estimated from gel filtration chromatography did not match any of these molecules. Cytochrome c has a MW of about 13 kDa while native hemoglobin has a MW of 64.5 kDa. The spectra of a cytosol sample, hemoglobin and cytochrome c were examined using UV/Visible spectrophotometry and are presented in Fig. 4.12. Since the spectra of hemoglobin and cytochrome c did not differ significantly, no conclusion could be drawn. Another qualitative test was performed. Samples of cytosol, hemoglobin and cytochrome c were titrated with about 2 M HCl from pH of about 7 to 4. Solutions of hemoglobin and cytosol turned from clear reddish to milky brownish, while the solution of cytochrome c remained clear even when more acid was added. Titration with about 2 M NaOH back to pH of about 7 brought both cytosol and hemoglobin samples to clear solution. On the basis of these brief tests as well as ruling out the possibility of cross-contamination between mitochondria and cytosol fractions, it was decided to continue our experiments with the assumption that the red color in the cytosol was due to hemoglobin.

It was then essential to develop an effective method for the removal of hemoglobin from cytosol. Two possibilities were considered: (i) elimination of

(a)



(b)



(c)

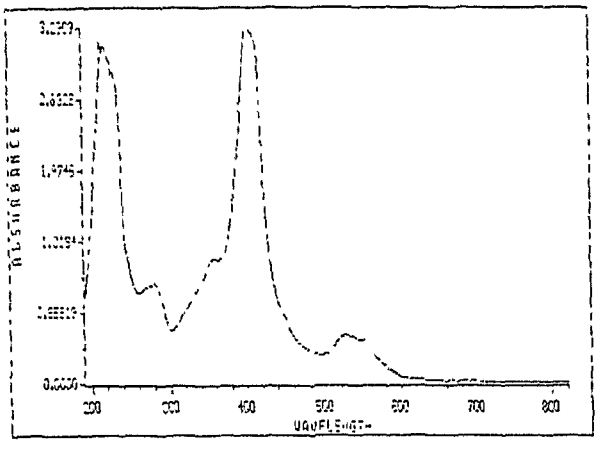


Fig.4.12. UV/visible spectrum of (a) cytosol; (b) hemoglobin; (c) cytochrome c.

hemoglobin from initial cytosol; and (ii) elimination of hemoglobin from the final peak eluted from the gel filtration column. The method applied by Yoshida and Watanabe [177] for the purification of phosphoglycerate kinase from red blood cells was slightly modified and applied to the crude cytosol. Indeed, the resulting precipitate was bright red, leaving only a pale yellow supernatant. We also anticipated that this step would enhance the overall purification ability of the developed procedure as other proteins might be removed from the sample along with hemoglobin.

The recovery of Se after a hemoglobin removal step with chloroform-ethanol is presented in Table 4.3. The overall recovery of Se after the experiment was 93% and 71% was found in the supernatant which will be referred to as the hemoglobin-free cytosol. Since only 8% of Se is dialysable from cytosol (Chapter 5, Table 5.1), 30% of Se that was lost during this step was most probably in a protein-bound form.

Table 4.3

Recovery of selenium in cytosol after removal of hemoglobin[a]

Sample	Se Total (μg)	Se Recovery (%)
Initial Cytosol	0.606 ± 0.021	(100)
Hemoglobin-free Supernatant	0.429 ± 0.028	70.8 ± 5.23
Hemoglobin Precipitate	0.133 ± 0.007	21.9 ± 1.38

[a] average of 4 determinations. Total recovery of Se: 92.7 ± 5.4%.

A sample of 100 mL of hemoglobin-free cytosol was subjected to ion-exchange chromatography on a DEAE-Sepharose column and proteins eluted by NaCl gradient to 0.75 M salt. Separate fractions were dialysed against water to remove NaCl, transferred to medium-size irradiation vials, dried under an IR lamp and analyzed for Se. The elution pattern along with the Se contents is shown in Fig. 4.13. First of all, a noticeable decrease in the absorbance was observed compared to the readings from the chromatography of the crude cytosol (Fig. 4.8). Another difference was that no significant amount of protein eluted from the column prior to the salt gradient. The Se profile was also different. This element was detected in the effluent practically as one broad peak eluted by up to 0.35 M NaCl. As a result, the subsequent anion exchange chromatography experiments with hemoglobin-free cytosol used a stepwise protein elution with 0.4 M NaCl. This slightly higher salt concentration was employed in order to ensure the elution of all Se from the column. Protein-containing fractions were pooled, concentrated about 20-fold with Aquacide, dialysed against 0.1 M HEPES buffer (pH 7.4) and applied to a gel filtration column. Samples were collected directly in medium-size irradiation vials, their absorbance at 280 nm measured and the samples were analyzed for Se by INAA after drying under an IR lamp. The elution profile of selenoproteins is shown in Fig. 4.14. Like in ion exchange chromatography, lower absorbance readings were observed compared to the untreated cytosol (Fig. 4.9). Some Se was again detected in the material that eluted in the void volume (about 130 ng) but a much higher Se content (about 400 ng in total) was present in the

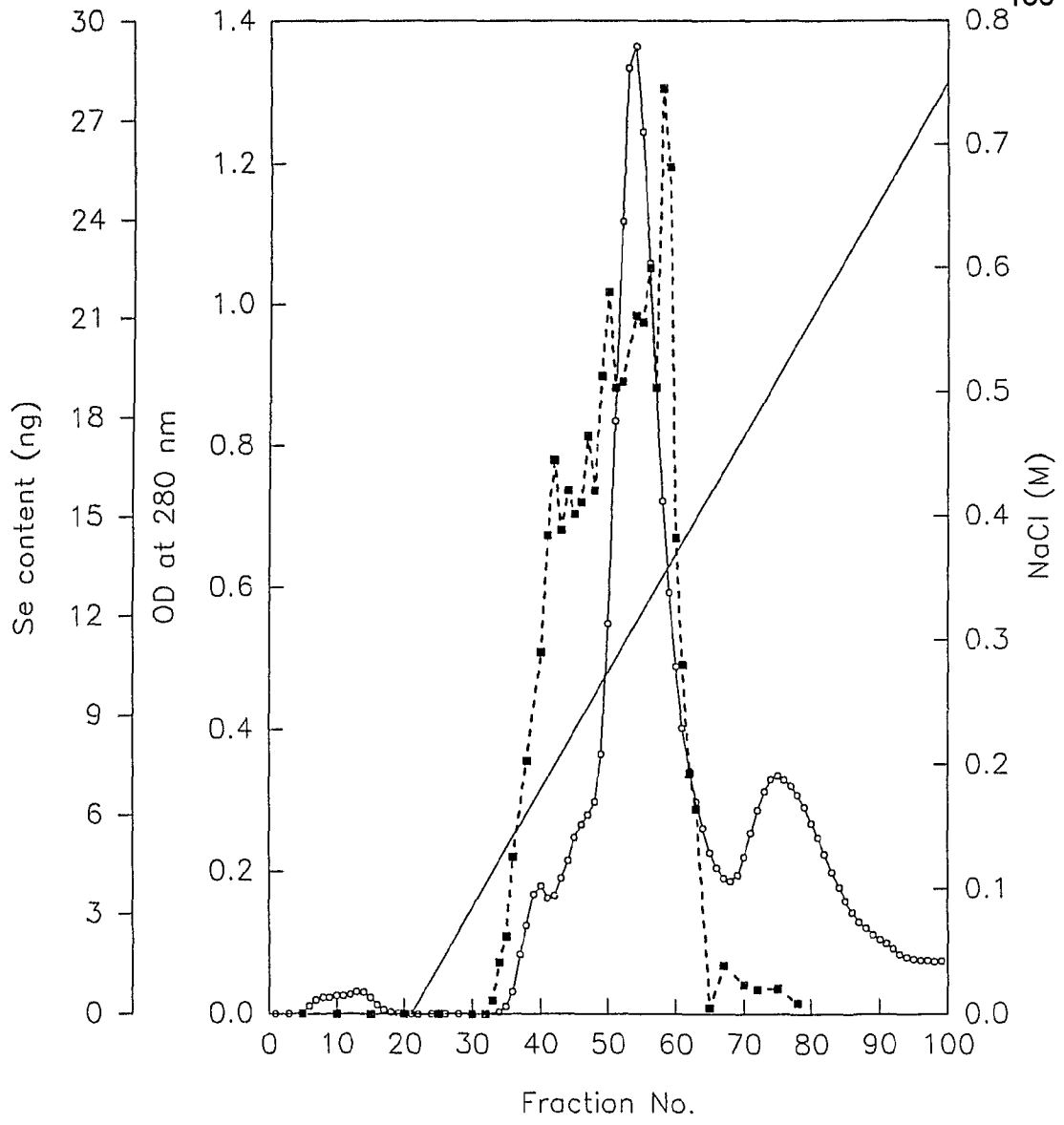


Fig. 4.13. Elution profile of hemoglobin-free cytosol on a DEAE-Sepharose column.

protein (—○—○—○—)

Se content (ng) (- -■- -■- -■- -)

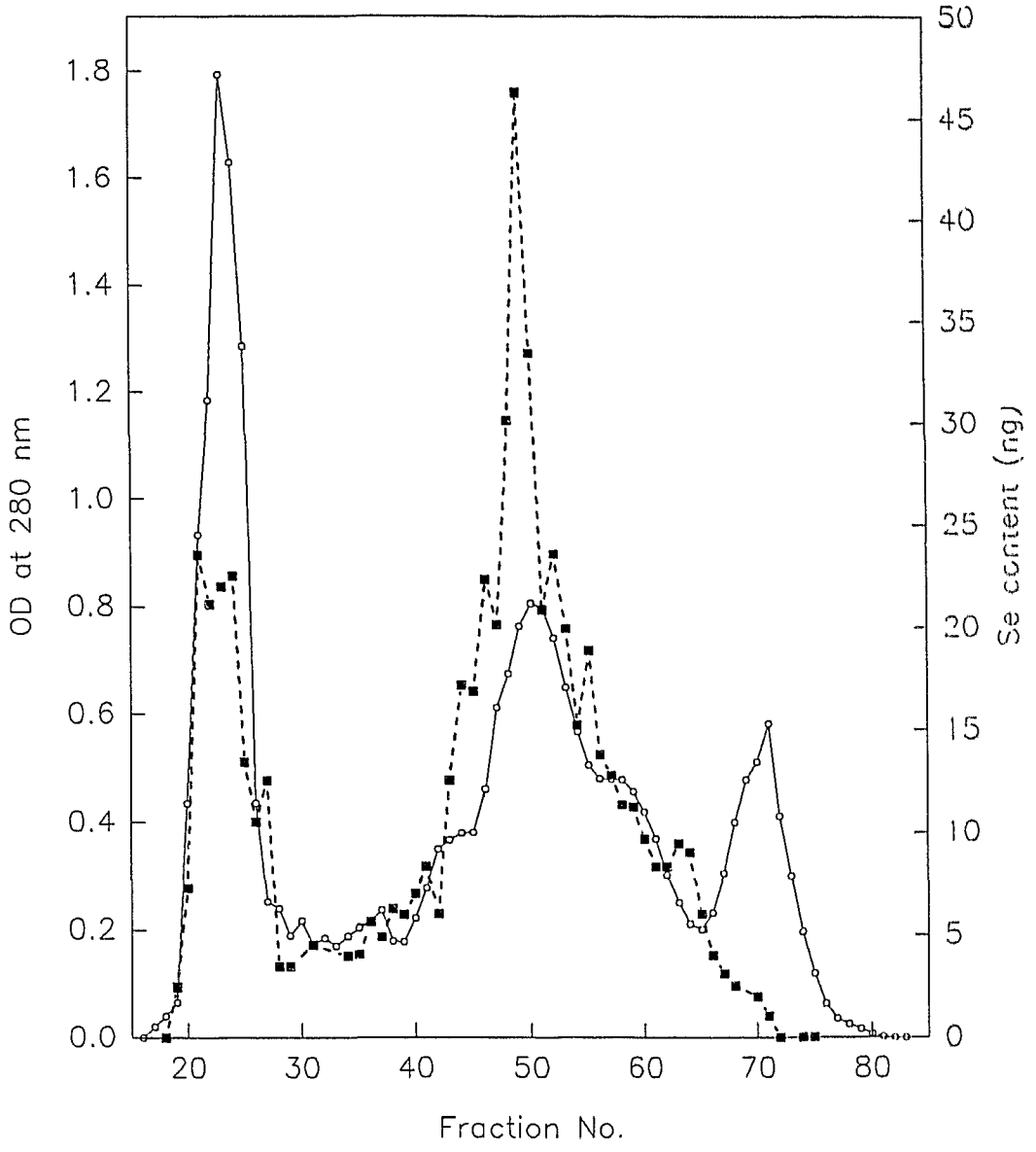


Fig. 4.14. Elution profile of hemoglobin-free cytosol (chromatographed on DEAE-Sepharose) on an Ultrogel AcA 34 column.

protein (—o—o—o—o—o—)

Se content (ng) (- -■- -■- -■- -)

second peak with an estimated MW of about 71 kDa (fraction No. 49). The peak was well resolved from other protein peaks although two shoulders, one on each side, were clearly visible. The highest absorbances of these shoulders corresponded to fraction No. 44 and fraction No. 58, respectively.

Polyacrylamide gel electrophoresis experiments in non-denaturing and denaturing systems were performed in order to determine the degree of purity and the MW of the purified protein (fraction No. 49, Fig. 4.14). This protein will be hereforth called Selenoprotein A1. Non-destructive PAGE was carried out using a 7.5% gel cast. Selenoproteins purified in four separate purification schemes from two different kidneys were analyzed and samples were loaded in duplicates. The electrophoretic pattern is presented in Fig. 4.15. It can be seen that one strong band is present in the 7.5% gel cast after staining. The existence of another protein is possible as very closely spaced two or three weak bands are present in the middle of the gel slab. This pattern is missing in the first two lanes.

A protein separation pattern obtained in SDS-PAGE on a 12.5% gel is shown in Fig. 4.16. A comparison of the cytosol and hemoglobin-free cytosol samples (lanes 8 and 9, respectively) shows the removal of many interfering proteins and smaller polypeptides during the ethanol-chloroform precipitation step. Only one band is present in samples of Selenoprotein A1 after staining. The MW of this protein was 70.6 ± 1.9 kDa (72.7 kDa, 70.0 kDa, and 69.1 kDa calculated from three different experiments). This value is in very good agreement with the value estimated by gel filtration chromatography (71 kDa).

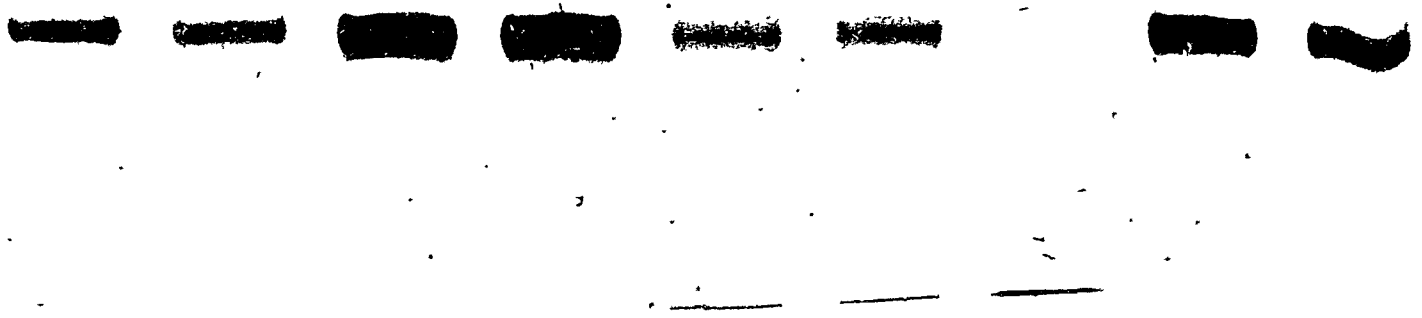


Fig. 4.15. Non-denaturing PAGE of purified Selenoprotein A1. Protein samples were obtained in four experiments and were loaded in duplicates.

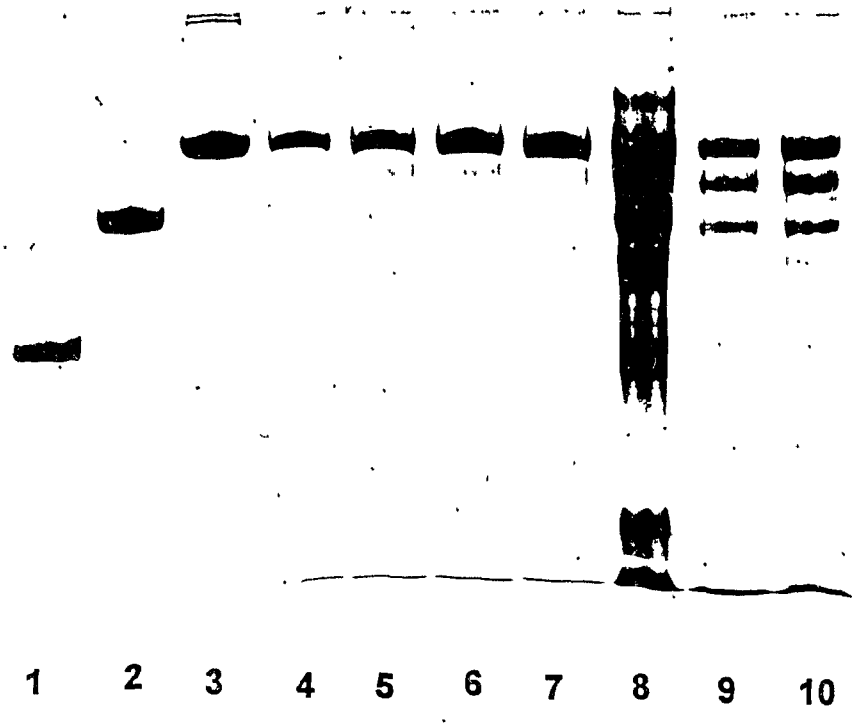


Fig. 4.16. SDS-PAGE of Selenoprotein A1, cytosol and hemoglobin-free cytosol.

Lane 1: Carbonic anhydrase, 29 kDa;

Lane 2: Albumin, egg, 45 kDa;

Lane 3: Albumin, bovine, 66 kDa;

Lanes 4 to 7: Selenoprotein A1 after the last step of purification;

Lane 8: Cytosol;

Lanes 9 and 10: Hemoglobin-free cytosol.

The isoelectric point of Selenoprotein A1 was determined by the isoelectric focusing experiment. The ampholyte of a pH range 3.5 - 10.0 was used and the pH gradient was determined using an LKB surface electrode. The pH of the electrofocused band was determined as 5.5 as presented in Fig. 4.17.

Although the protein was found to be pure after the applied purification procedure, it still remained to be determined if it is a genuine selenoprotein. One of the best evidences for the existence of selenoprotein is the presence of selenocysteine in the protein polypeptide. However, as already mentioned in the literature survey, various research groups perform different tests which, in their opinions, prove that Se is covalently bound to the molecule. Many of them claim [40], [41] that if Se-protein bond can withstand a rigorous treatment with SDS, 2-mercaptoethanol and heating prior to the SDS-PAGE then the molecule is really a metalloprotein. This approach was adopted here although our studies were more difficult then those found in the literature. In the reported studies, ^{75}Se was injected or fed to animals and the radioactivity was measured in post electrophoresis bands. This method of detection was very sensitive and required as little as a few μL of protein sample for precise measurements. In our approach, the idea to use radiotracers was rejected right from the beginning of the whole project. In order to detect Se in gels the experiment would have to be run at a much larger scale which was not feasible due to lack of proper instrumentation. The option of performing several SDS-PAGE experiments, isolating protein band (corresponding to the selenoprotein) from the rest of the gel and irradiating as

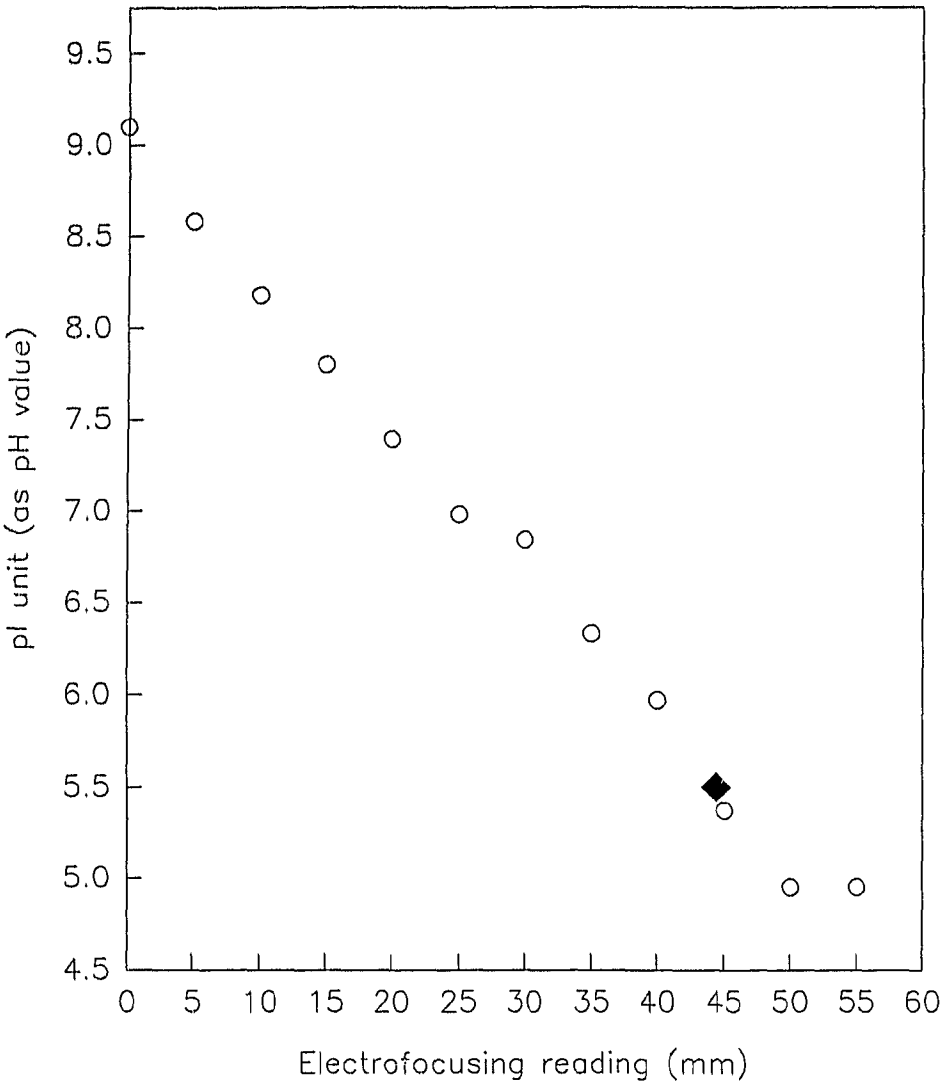


Fig. 4.17. Isoelectric focusing of Selenoprotein A1.

electrode readings (○)

Selenoprotein A1 (◆)

many accumulated samples as possible was investigated. However, no Se was detected by INAA. This negative results could be due to insufficient amount of selenoprotein collected for irradiation.

Total protein content and total Se content after each step of purification was measured in order to calculate the Se-to-protein ratio. Protein was first assayed by Lowry's method. This is one of the most widely used assays for protein quantitation due to its sensitivity and simplicity. Its main disadvantage is its lack of specificity. Many substances like ammonium sulfate, SDS, sucrose and various buffers are known to interfere with this method. They may cause an increase in the absorbance of the reagent blank, decrease in the absorbance of the protein, or both. Samples were dialysed against 0.001 M Tris buffer before the assay in order to eliminate possible interfering effects. The results, however, did not indicate any increase in the Se-to-protein ratio. It was assumed that the protein determination step produced erroneous results. The protein determination method was therefore changed to the Bio-Rad method as this assay has been suggested as a replacement of Lowry's method for most research applications [184]. A test of comparison of both methods was performed. Samples of cytosol and its fractions after stepwise ammonium sulfate precipitation were analyzed by both procedures. Procedure for the precipitation step is described in detail in Chapter 5, Section 5.2.3. The results are presented in Table 4.4. The same protein standard, *i.e.* bovine serum albumin, was used in both assays. As can be seen, the protein concentrations determined using the Lowry method were approximately

Table 4.4

Protein content in cytosol and ammonium sulfate precipitates.

Comparison of Lowry and Bio-Rad methods

Fraction	Lowry Method[a]		Bio - Rad Method[a]	
	Protein Concentration (mg ml^{-1})	Recovery in Fractions (%)	Protein Concentration (mg ml^{-1})	Recovery in Fractions (%)
Initial Cytosol	3.41 ± 0.11	(100)	1.88 ± 0.06	(100)
1 M ppt.	3.18 ± 0.23	4.7 ± 0.4	1.26 ± 0.07	3.6 ± 0.2
2 M ppt.	11.31 ± 0.56	37.3 ± 2.2	5.81 ± 0.37	34.8 ± 2.5
3 M ppt.	9.86 ± 0.38	32.5 ± 1.6	5.09 ± 0.12	30.5 ± 1.2
4 M ppt.	4.92 ± 0.21	17.50 ± 0.05	2.58 ± 0.09	16.7 ± 0.8
Final Supernatant	0.050 ± 0.004	1.8 ± 0.15	N/D	-

[a] average of 5 determinations.

N/D = not detected.

twice as high as those by the Bio-Rad method in all fractions. On the other hand, the calculated percent distributions of total protein in particular fractions were similar in both assays. This might suggest an interference problem in the Lowry method. Samples after each purification step were then reanalyzed using the Bio-Rad assay, but still no increase in the purification factor was observed.

Several factors may account for these results. First, the ethanol-chloroform precipitation might have created too drastic conditions for the binding of Se to protein. As a consequence, only part of Se was still associated with the protein peak and could therefore be determined by INAA. As a result, this would not give any enrichment in Se-to-protein ratio as purification steps continued. The other explanation of the negative results is that the error would be in the protein assay. Interfering substances might still have been present in tested samples and caused an increase in the absorbance readings. In fact, other studies done on Se-proteins in our laboratory by Jayawickreme [70] also gave an inaccuracy in the Bio-Rad method.

4.4.2.1 Summary of the Purification of Selenoprotein A1

The purification of a Se-containing protein from cytosol involved the sequential use of a chloroform-ethanol mixture to remove the major interfering protein(s) from the sample, chromatography on DEAE-Sepharose and gel filtration chromatography on Ultrogel AcA 34. The MW and pI of the purified protein (referred to as A1) determined by SDS-PAGE and isoelectric focusing were 70.6

± 1.9 kDa and 5.5, respectively. Although in the electrophoresis experiment the protein was found to be free from contaminants, no increase was found in the Se-to-protein ratio in each subsequent purification step. The following reasons could account for this observation: (i) denaturation of protein during chloroform-ethanol treatment which resulted in the loss of Se; and (ii) methods used for protein determination gave higher values for protein concentration due to the presence of interfering substances in the samples.

4.4.3 Purification of Selenoproteins P1 and P2

As the first modification to the previous procedure, it was decided to measure protein concentration using the BCA (bicinchoninic acid) protein assay reagent. This method, apart from its simplicity, offers better sensitivity and permits the accurate measurement in the presence of detergents and other substances interfering otherwise with the Lowry method [185].

From the results of previous experiments it was suspected that chloroform-ethanol precipitation of hemoglobin from cytosol might cleave the Se-protein bond which in turn resulted in the partial loss of Se from the purified protein. Before applying any chromatographic separation it was desirable to include a fractionation step that would eliminate most proteins other than Se-proteins of interest. Although Froom [181] ruled out the possibility of using ammonium sulfate precipitation as an initial step in the fractionation scheme, this method is widely used by others to study Se-proteins in various biological samples [142], [143],

[157], [162]. It was decided to evaluate the applicability of this method in our studies.

Proteins from the cytosol fraction were subsequently precipitated from the solution using the procedure described in Chapter 5, Section 5.2.3. The Se and protein contents were then determined and results are presented in Table 4.5. As can be seen, 2 M salt precipitated 55% of total Se and gave the highest Se-to-protein ratio, *i.e.* the highest enrichment of Se-proteins. For the purpose of INAA, precipitates were dissolved in water, dialysed and then freeze-dried. For future use in enzyme assay or in biochemical separation steps, precipitates were dissolved in the appropriate buffers and then dialysed against these buffers. In order to prevent potential denaturation, the time of dialysis was kept to a minimum by frequent changes of the dialysing media. No significant precipitation during dialysis was observed.

In the next step the glutathione peroxidase activity was assayed in the fractionated cytosol samples (as described in Chapter 3, Section 3.2.6). As seen in Table 4.6, the highest activity, highest specific activity and 65% of total activity of the enzyme were found in the 3-M fraction. The 2-M fraction, on the other hand, accounted for less than 12% of the total activity. These results motivated us to replace the chloroform-ethanol mixture with 2 M ammonium sulfate as a precipitating agent in the first step of purification.

Table 4.5

Selenium in cytosol fractionated by ammonium sulfate precipitation[a]

Fraction	Se recovery (%)	Se/Protein
1 M ppt.	5.8 ± 0.4	1.23 ± 0.13
2 M ppt.	55.4 ± 5.0	1.49 ± 0.16
3 M ppt.	26.3 ± 1.1	0.81 ± 0.05
4 M ppt.	6.7 ± 0.5	0.37 ± 0.03
Final Supernatant	0.30 ± 0.02	0.17 ± 0.02

[a] average of 7 determinations. Total recovery of Se: 94.2 ± 5.2%.

Cytosol was subjected to ammonium sulfate fractionation as described in Section 4.2.2.1. Samples were then applied to a DEAE-Sepharose column and proteins eluted stepwise with 0.4 M NaCl. Protein (P) and Se, and protein and Se/P ratio (μg Se/mg P) elution patterns are presented in Figs. 4.18 and 4.19, respectively. The largest protein peak was divided into four sections and the following fractions were combined for this purpose: 41-44 (Section I, S-I); 45-50 (S-II); 51-55 (S-III); and 56-62 (S-IV). Insignificant amount of Se eluted from the column without binding and the highest Se-to-protein ratios were found in S-I and

Table 4.6

Glutathione peroxidase activity in cytosol fractionated by ammonium sulfate precipitation[a]

Fraction	Protein (mg mL^{-1})	Activity (u mL^{-1})	Specific Activity	Total Activity (u)	Recovery of Activity (%)
Cytosol	3.41±0.11	15.02±0.84	4.40±0.28	300.0	(100)
1 M ppt.	3.18±0.16	0	0	0	0
2 M ppt.	11.31±0.74	12.21±1.45	1.08±0.14	35.0	11.7
3 M ppt.	9.86±0.79	88.32±4.78	8.96±0.75	194.0	64.7
4 M ppt.	4.92±0.39	19.75±1.13	4.01±0.38	35.6	11.9
Final Supernatant	0.046±0.002	0.10±0.02	2.17±0.44	2.2	0.7

[a] average of 5 determinations.

Total recovery of enzymatic activity: 89%.

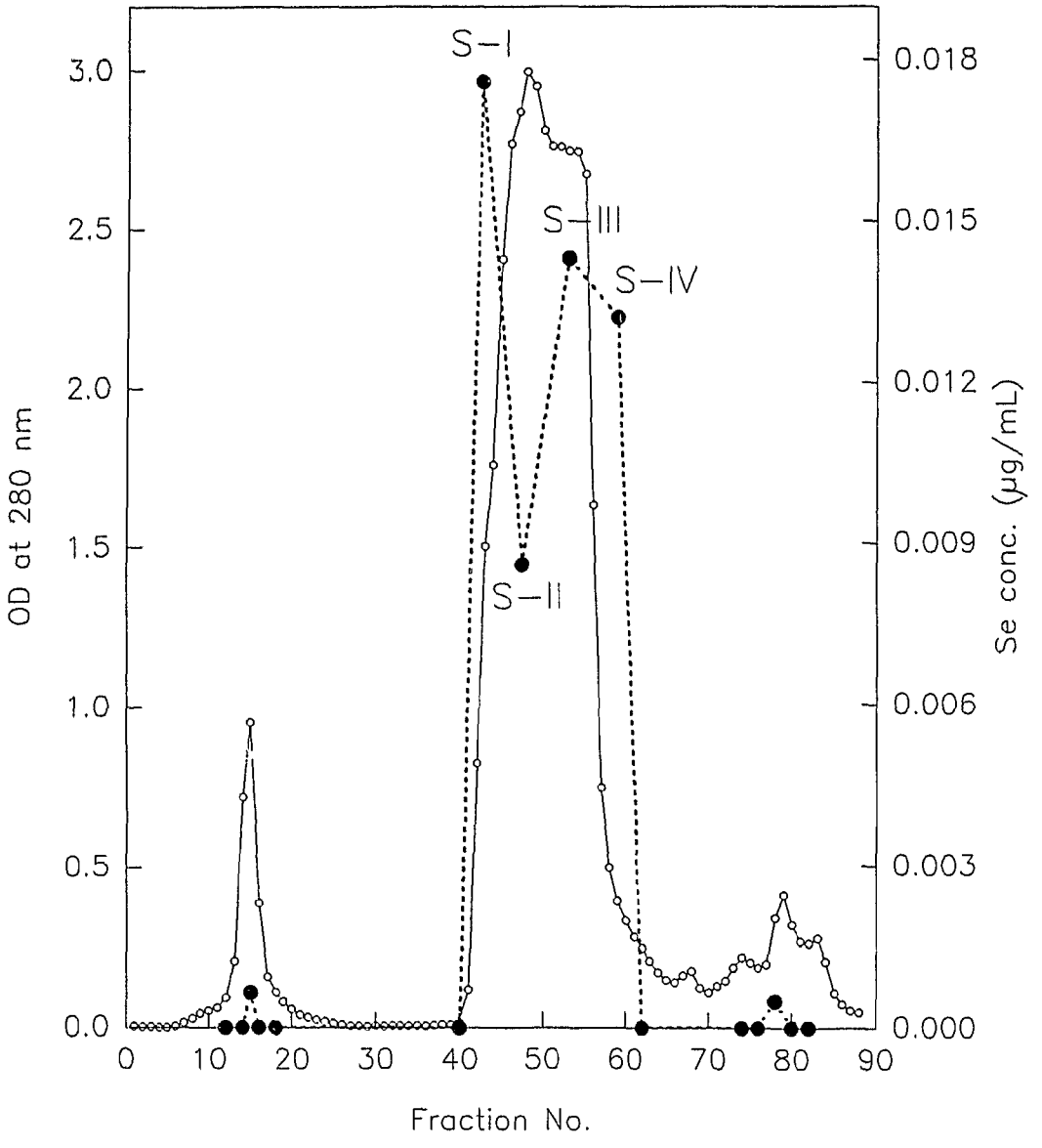


Fig. 4.18. Elution profile of cytosol proteins (precipitated with 2 M $(NH_4)_2SO_4$) on a DEAE-Sepharose column.

protein (–o–o–o–o–)

Se concentration (µg mL^{-1}) (–●–●–●–)

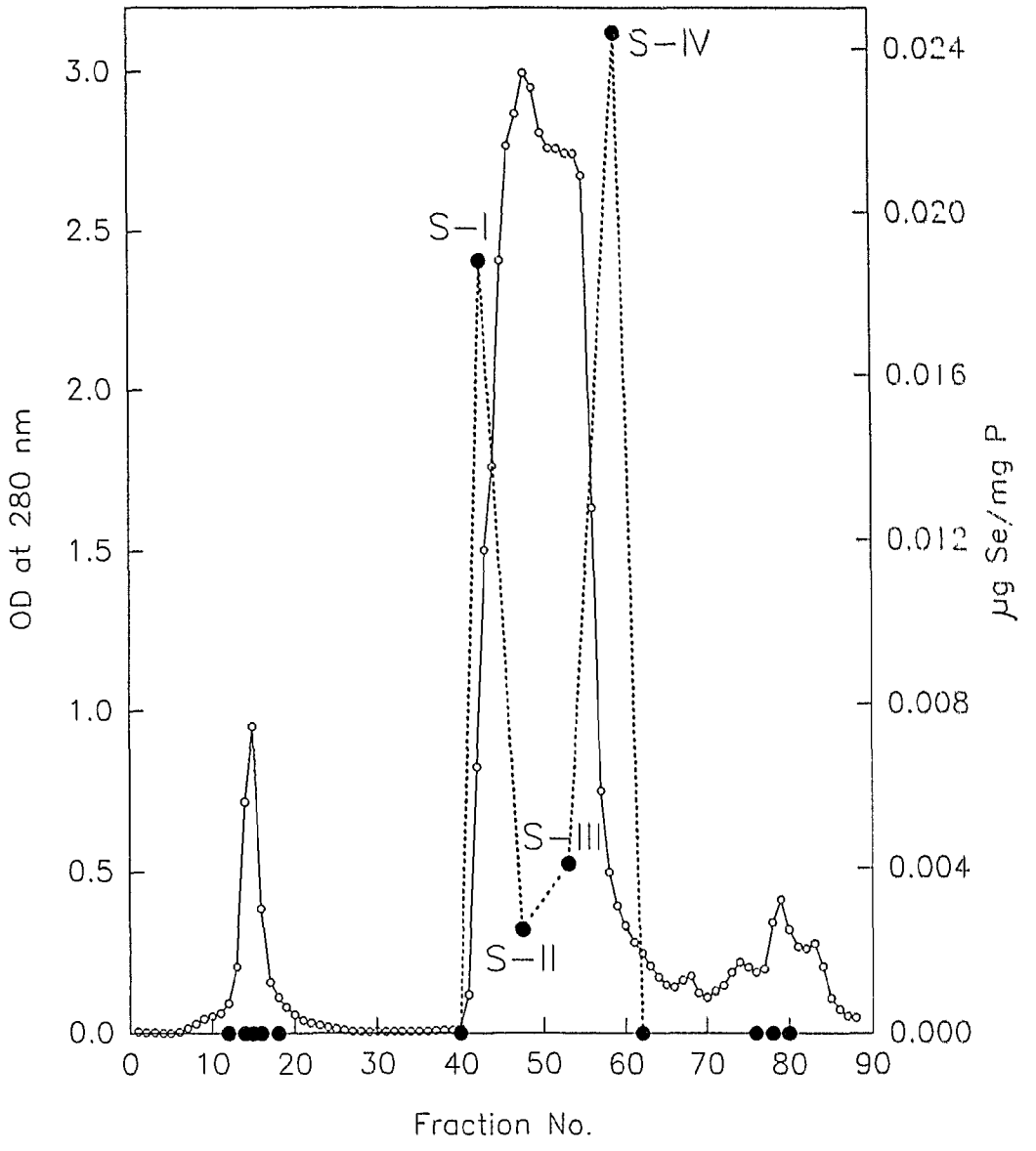


Fig. 4.19. Elution profile of cytosol proteins (precipitated with 2 M $(NH_4)_2SO_4$) on a DEAE-Sepharose column.

protein (–o–o–o–o–)

Se / P (···●···●···●)

S-IV (Table 4.7) as 18.8 and 24.4, respectively. It was decided to further purify these two fractions.

Table 4.7

Selenium and protein in peak eluted from a DEAE-Sepharose column

Sample	Se conc. ($\mu gSe\ mL^{-1}$)	Protein conc. ($mg\ P\ mL^{-1}$)	Se / Protein (ppm)
S-I (Fractions 41-44)	0.0176	0.936	18.8
S-II (Fractions 45-50)	0.0086	3.480	2.5
S-III (Fractions 51-55)	0.0143	3.495	4.1
S-IV (Fractions 56-62)	0.0132	0.540	24.4

Before applying the DEAE fractions to a gel filtration column, a considerable reduction of their volumes was needed. It was observed in previous experiments that samples became highly viscous after concentration with Aquacide. This viscosity could be influenced by Aquacide. Such a sample would sometimes cause the solvent flow to slow down significantly on the column. It was decided to use ammonium sulfate precipitation instead of Aquacide to concentrate proteins.

Proteins in samples S-I and S-IV from ion exchange chromatography

experiment were precipitated with 3 M ammonium sulfate, the resulting precipitates dissolved in a minimum volume of 0.1 M HEPES buffer (pH 7.4) and applied separately on a gel filtration column. Protein and Se elution profiles of each sample are presented in Figs. 4.20 and 4.21, respectively. In order to detect Se, three or four fractions had to be combined for irradiation. Proteins from sample S-I separated on the column into three, well resolved peaks (Fig. 4.20). A significant amount of material eluted in the column void volume. All three peaks contained Se and the highest Se concentration was associated with the second protein peak (about 0.0015 μg mL^{-1}). Proteins from sample S-IV also separated into three peaks (Fig. 4.21). The amount of material that eluted from the column in a void volume was very small. The third and highest peak contained two shoulders on its low-MW side. It was also the highest Se peak with the concentration up to 0.003 μg mL^{-1}. The results indicate that the sections S-I and S-IV eluted from ion exchange chromatography (Fig. 4.19) were mixtures of two selenoproteins which could be resolved on an Ultrogel column. In addition, a Se peak eluted in the column void volume. The first section of the peak, *i.e.* S-I (the one that eluted earlier), contained mostly protein of high MW weight (which will be named hereforth Selenoprotein P1), while the last section of the peak, *i.e.* S-IV (the one that eluted later from the column), contained protein of low MW (which will be named hereforth Selenoprotein P2). Earlier elution of P1 from the anion exchange column might also suggest a higher pI compared to protein P2.

The molecular weights and degrees of purity of these two selenoproteins

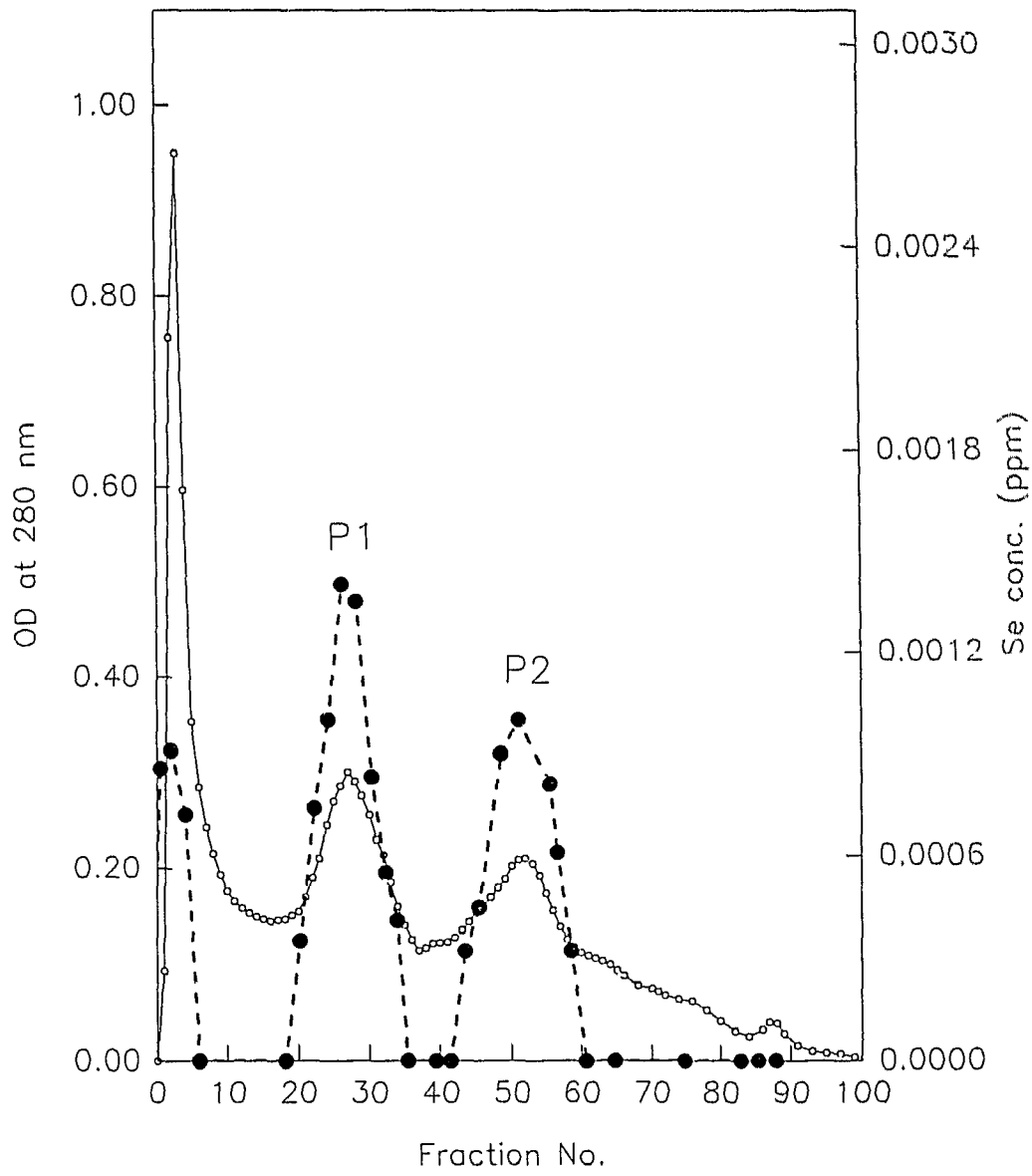


Fig. 4.20. Elution profile of S-I on an Ultrogel AcA 34 column.

protein (—o—o—o—o—)

Se concentration (ppm) (--●--●--●-)

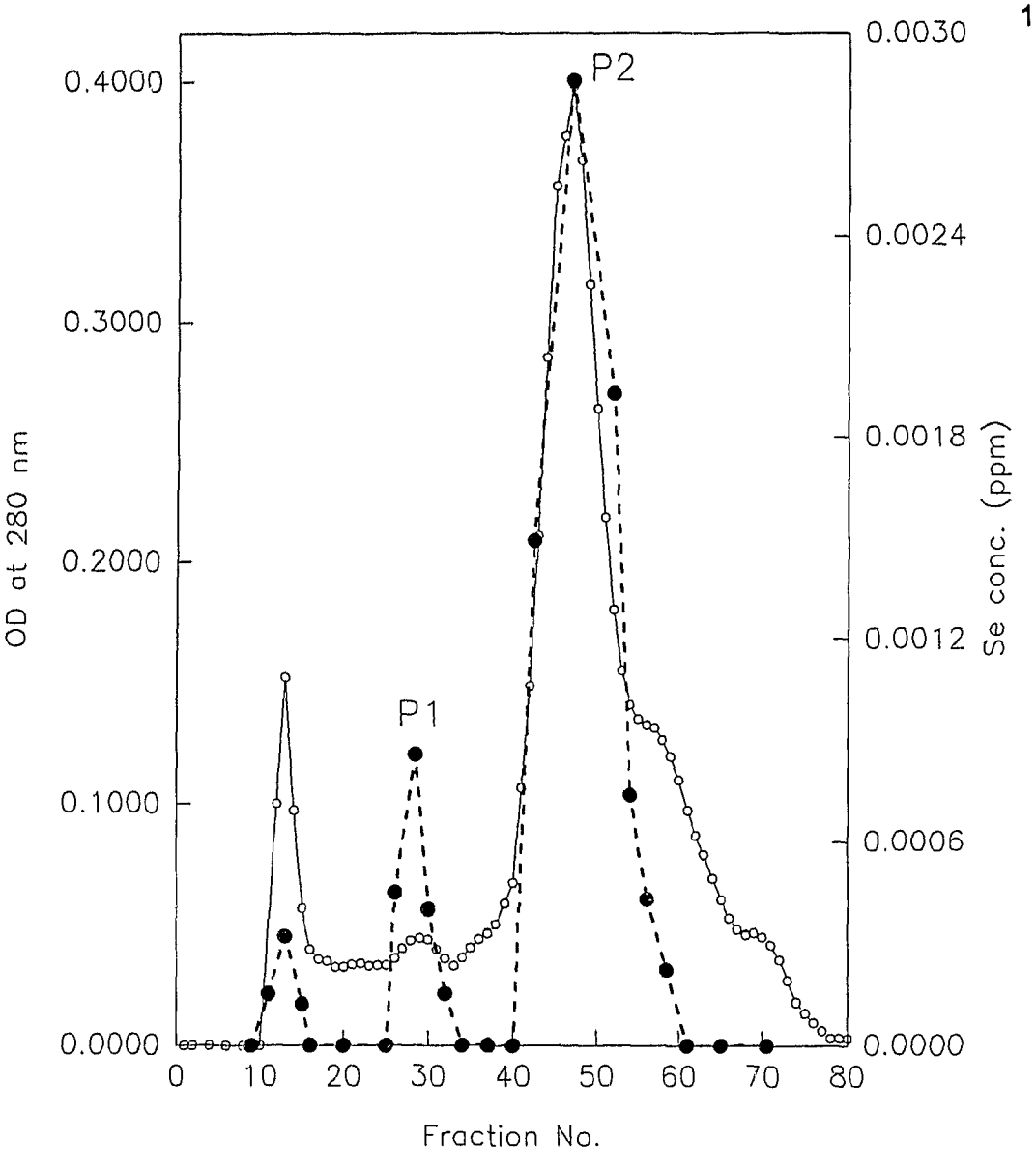


Fig. 4.21. Elution profile of S-IV on an Ultrogel AcA 34 column.

protein (-o-o-o-o-)

Se concentration (ppm) (--●--●--)

were determined by SDS-PAGE. Fractions corresponding to the highest Se concentrations were pooled and applied to the sample wells in the gel plate. The electrophoresis patterns of two peaks P1 and P2 are presented in Fig. 4.22. Both proteins gave only one band in the presence of SDS which could be taken as proof of their purity. The MW of the smaller protein (P2) was determined as 48.0 ± 1.0 kDa and the larger one (P1) as 62.0 ± 0.3 kDa.

An analytical isoelectric focusing experiment was performed in order to determine the pI values of the purified selenoproteins. Ampholine of 3.5-10.0 range was used in the preliminary experiment and the pH gradient was determined on the basis of distances migrated by protein standards. A concentration of bands in the region of pH 5.0 prompted us to apply Ampholine of a narrower, acidic pH range, *i.e.* pH 4.0 - 6.5. The resulting pattern is shown in Fig. 4.23. From left to right, the following samples were applied to the gel wells: lanes 1 - 4: protein standards; lane 5: Selenoprotein P1 from sample S-IV (Fig. 4.21); lane 6: Selenoprotein P2 from sample S-IV (Fig. 4.21); lane 7: Selenoprotein P1 from sample S-I (Fig.4.20); and lane 8: Selenoprotein P2 from sample S-I (Fig 4.20). The electrofocusing pattern of the selenoproteins is not very clear due to too low protein concentration of the samples loaded on to the gel. However, in lanes 5 and 7 (P1) one sharp band of a protein at 30 mm from the cathode where the pH = 5.4 is visible. In lanes 6 and 8 (P2) there is one strong band present at a distance of 34 mm from the cathode where the pH = 4.9. The plot of pH *vs* the distances migrated by proteins is presented in Fig. 4.24. The results obtained are

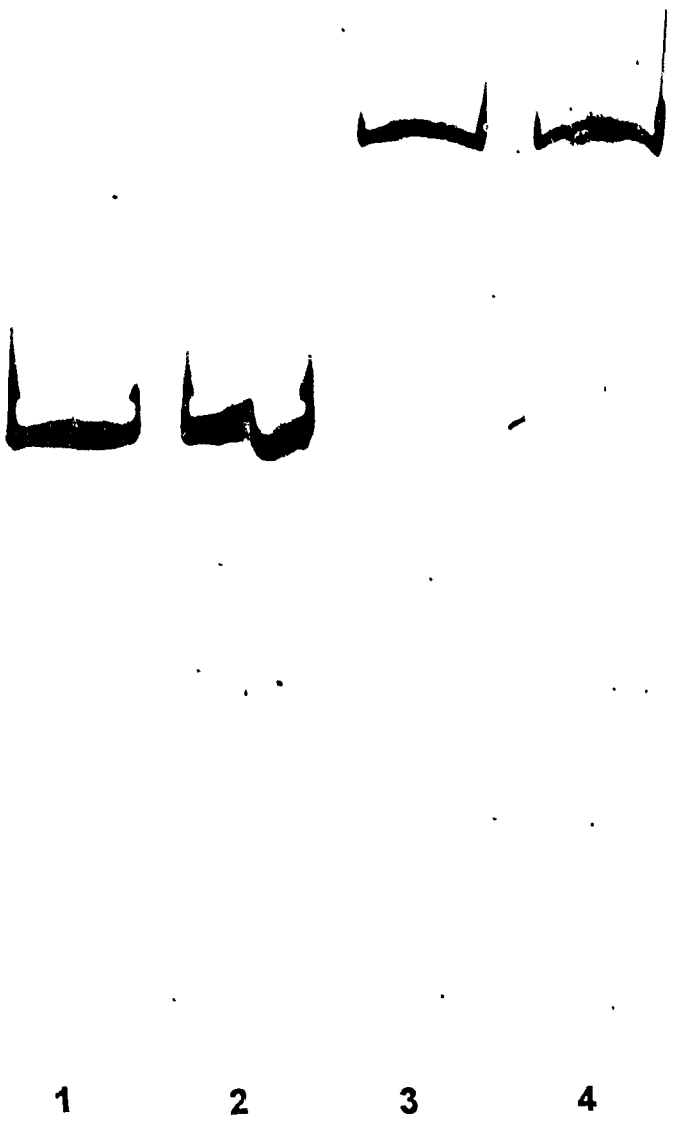


Fig. 4.22. SDS-PAGE of Selenoproteins P1 and P2.

Lanes 1 and 2: Selenoprotein P2;

Lanes 3 and 4: Selenoprotein P1.

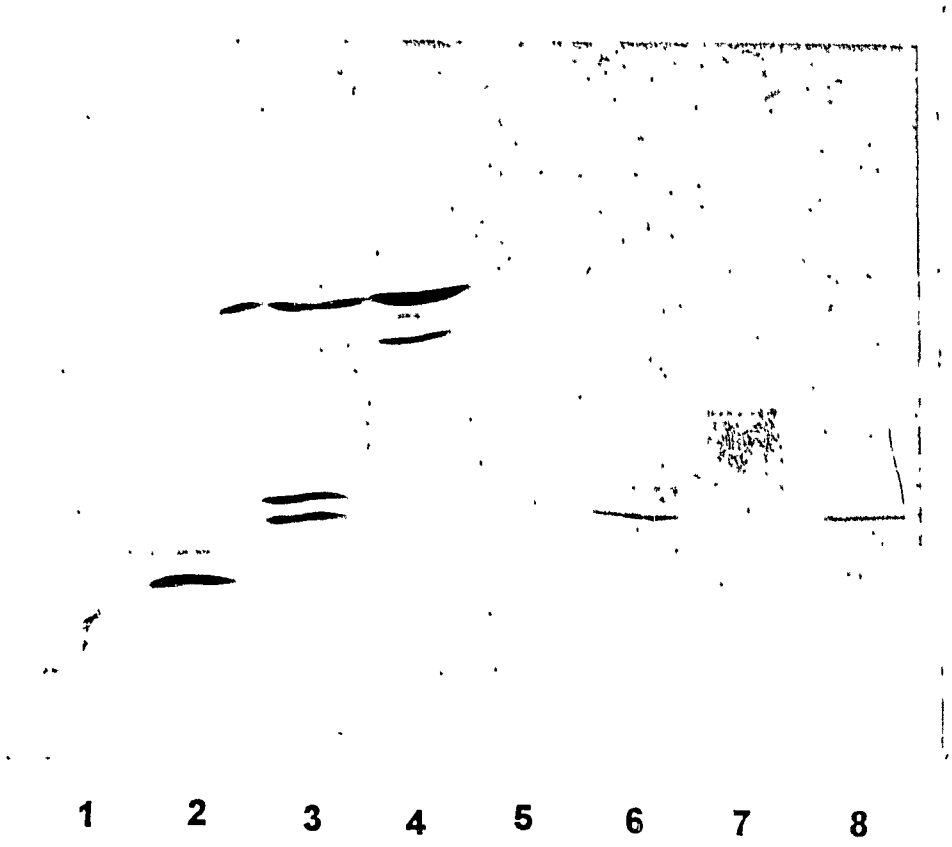


Fig. 4.23. Isoelectric focusing of Selenoproteins P1 and P2.

Lane 1: Glucose oxidase, pI 4.2;
Lane 2: Tripsin inhibitor, pI 4.6;
Lane 3: β-lactoglobulin, pI 5.1;
Lane 4: Carbonic anhydrase, pI 5.9;
Lane 5: Selenoprotein P1 from sample S-IV;
Lane 6: Selenoprotein P2 from sample S-IV;
Lane 7: Selenoprotein P1 from sample S-I;
Lane 8: Selenoprotein P2 from sample S-IV.

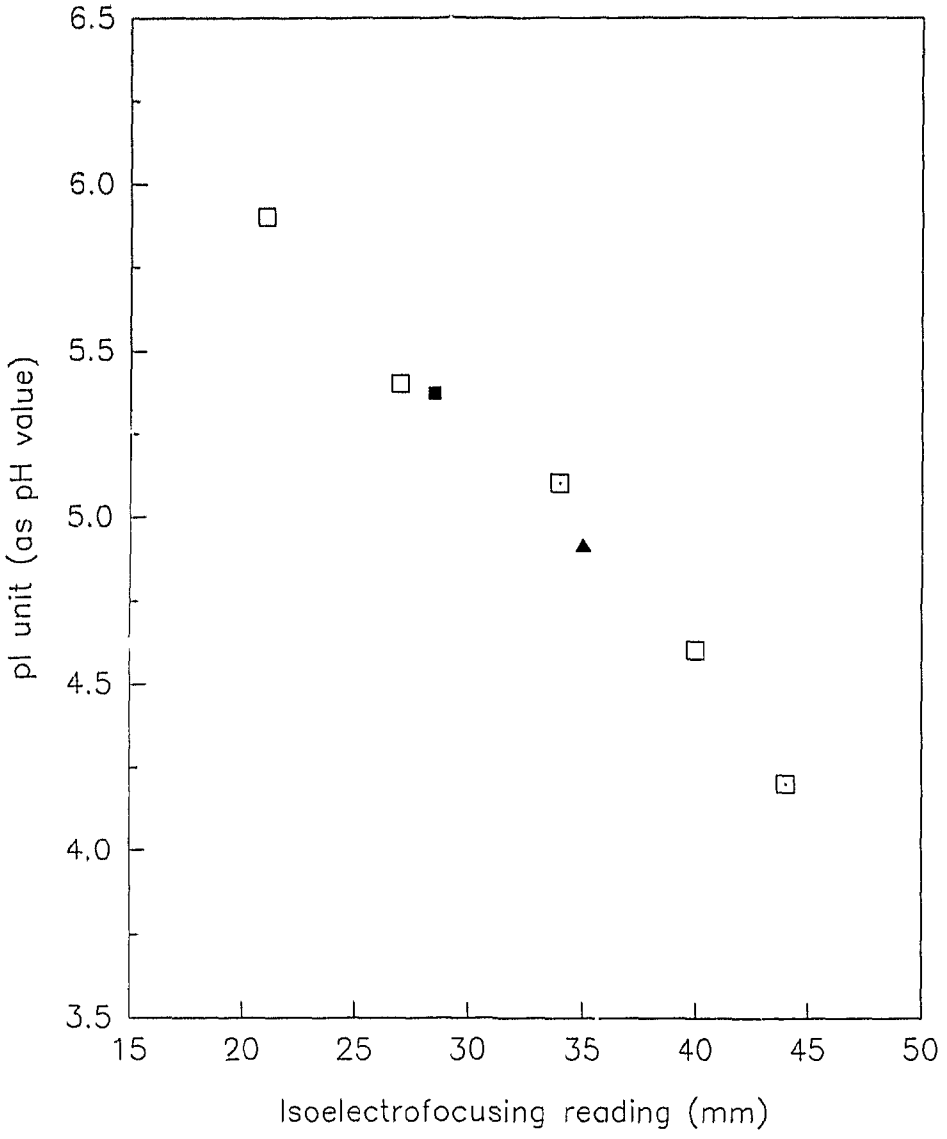


Fig. 4.24. Isoelectric focusing of Selenoproteins P1 nad P2.

protein markers (□)

Selenoprotein P1 (■)

Selenoprotein P2 (▲)

in a good agreement with earlier findings from ion exchange chromatography. The protein that eluted earlier from the column in the ion exchange chromatography (P1 from S-I) has a higher pI than the protein that eluted later (P2 from S-IV).

One can distinguish more than one band present in each purified protein sample. One diffuse band is visible below the sharp band in lanes 5 and 7; two closely spaced weak bands are present above and one or two very weak bands below the main band in lanes 6 and 8. It is frequently reported in the literature that protein samples which appear to be homogenous when tested by other techniques can often be separated into several components by IEF because of a high resolution of this technique. The following reasons apart from sample heterogeneity can account for this type of pattern: (i) binding of amphclytes to proteins; (ii) pH-dependent conformational changes of proteins; (iii) protein denaturation; (iv) existence of a protein as inherently heterogeneous mixture of isoelectric isomers; and others. As can be seen with pure protein standards, three out of four of them consist of more than one isomer. Trypsin Inhibitor, pI 4.6 (lane 2) consists of two bands; β-Lactoglobulin, pI 5.1 (lane 3) consists of two or possibly three bands; Carbonic Anhydrase, pI 5.9 (lane 4) consists of three bands. The diffuse bands in lanes 5 and 7 could arise from the association of protein with polyampholytes.

Although none of the purified proteins had a MW equal or similar to that of glutathione peroxidase, the activity of this enzyme was assayed in both proteins in order to completely confirm its absence. No activity was detected in either

Selenoprotein P1 or Selenoprotein P2.

A summary of the purification procedure for Selenoprotein P1 is presented in Table 4.8. Partial separation of this protein from Selenoprotein P2 was observed initially after a DEAE-Sepharose chromatography as Selenoprotein P1 eluted ahead of Selenoprotein P2. Protein yield and Se yield in a purified P1 were 0.0012% and 0.17%, respectively. The MW of this protein was determined as 62.0 ± 0.3 kDa. Assuming this MW of 62 kDa and 1 g-atom of Se per mole of protein, then 0.28 mg of purified protein should contain 0.35 µg of Se. A significant loss of protein and Se was observed after gel filtration chromatography which showed slow flow rate of solvent through the column. These observations suggested a protein precipitation and denaturation on the column.

A summary of the purification procedure for Selenoprotein P2 is presented in Table 4.9. Both the ion exchange and gel filtration chromatography steps turned out to be more advantageous for this protein than for Selenoprotein P1. Protein yield and Se yield in a purified P1 were 0.0015% and 0.41%, respectively. The molecular weight of this protein was determined as 48.0 ± 1.0 kDa. Assuming this MW of 48 kDa and 1 g-atom of Se per mole of protein, then 0.34 mg of purified protein should contain 0.55 µg of Se. Again, as in the case of P1, low protein and Se yields were observed after a gel filtration chromatography, and it is suggested that Selenoprotein P2 could also partially precipitate and be lost during that step.

Table 4.8. Purification of Selenoprotein P1

Purification Step	Protein (mg)	Protein Yield (%)	Selenium (µg)	Se/Protein (ppm)
Homogenization	22 935	(100)	71.1	3.10
Ultracentrifugation	2 984	13	3.70	1.24
2 M $(NH_4)_2SO_4$ Precipitation	635	2.8	2.05	3.23
DEAE-Sepharose	28.1	0.12	0.53	18.8
Gel Filtration (Ultrogel AcA 34)	0.28	0.0012	0.12	429

Table 4.9. Purification of Selenoprotein P2

Purification Step	Protein (mg)	Protein Yield (%)	Selenium (µg)	Se/Protein (ppm)
Homogenization	22 935	(100)	71.1	3.10
Ultracentrifugation	2 984	13	3.70	1.24
2 M $(NH_4)_2SO_4$ Precipitation	635	2.8	2.05	3.23
DEAE-Sepharose	28.1	0.12	0.69	24.4
Gel Filtration (Ultrogel AcA 34)	0.34	0.0015	0.29	853

4.4.3.1 Summary of Purifications of Selenoproteins P1 and P2

The purification of two selenoproteins P1 and P2 from cytosol involved the sequential use of 2 M ammonium sulfate fractionation of cytosol, chromatography on DEAE-Sepharose and gel filtration chromatography on Ultrogel AcA 34. The MWs and pI values of these proteins were: 62.0 ± 0.3 kDa and 5.4 for Selenoprotein P1 and 48.0 ± 1.0 kDa and 4.9 for Selenoprotein P2, respectively. SDS-PAGE gave one band for each protein which demonstrated their purity as well as the presence of only one subunit. Partial losses of selenoproteins P1 and P2 were observed after gel filtration chromatography, which were likely due to the precipitation of the protein on the column.

4.5 SUMMARY

Selenium and selenoproteins in bovine kidney cytosol fraction were investigated using a combination of several bioanalytical and analytical techniques. At least 5 Se-containing proteins were detected. Three selenoproteins, named Selenoprotein A1, Selenoprotein P1 and Selenoprotein P2, were purified.

Kidney is not a widely studied organ despite its high Se level which cannot be explained in terms of glutathione peroxidase activity. Studies on Se in bovine kidneys are not reported in the literature. Other investigations were done on rat kidneys where the animals were given usually elevated Se doses in a diet or injected with ^{75}Se radiotracer. Motsenbocker and Tappel [43] reported the presence of a 75 kDa Se-protein in rat kidney. The MW was estimated from gel

filtration chromatography and the protein was shown to be chromatographically different from glutathione peroxidase. The protein contained one subunit and selenocysteine was detected in amino acid analysis experiment. However, they did not attempt purification of this protein. We, on the other hand, purified the selenoproteins from naturally Se-balanced bovine kidney and characterized them more extensively. Therefore, no relationship between the 75-kDa protein and protein A1 (MW of 71 kDa, pI of 5.5) purified in this study can be established. Two other selenoproteins, namely P1 (MW 62 kDa, pI 5.4) and P2 (MW 48 kDa, pI 4.9) appear to be novel proteins and not reported in the literature previously. Jayawickreme (unpublished data) indicated the presence of a 65 kDa (± 30%) protein in bovine kidney supernatant. However, his studies were not pursued any further towards purification of this protein so no direct comparison can be made.

5. STUDIES ON SELECTED PROTEIN-BOUND TRACE ELEMENTS IN THE CYTOSOL FRACTION

5.1 INTRODUCTION

The main purpose of this research project was to investigate Se-binding proteins in bovine kidneys. A few other elements were also studied since NAA allows for simultaneous multi-element determination. In this chapter, the preliminary studies on selected trace elements in the cytosol fraction of bovine kidneys are presented. An investigation on the behavior of several major and trace elements during dialysis and ammonium sulfate precipitation was carried out. Additionally, anion exchange chromatography was applied to study protein-bound As, Cu, Mn, V and Zn. Similar studies have been performed in the past [70] in our laboratory on the supernatant fraction of bovine kidneys and the results are compared and discussed here.

5.2 EXPERIMENTAL

5.2.1 Chemicals and Reagents

All chemicals used were purchased from the Sigma Chemicals, USA, unless otherwise noted. Ammonium sulfate, ammonium acetate, Tris, DEAE-Sepharose (Pharmacia) and Aquacide (CALBIOCHEM) were analyzed by INAA for the presence of trace element contaminants. The gel, used in ion exchange

chromatography, was equilibrated with 0.1 M Tris/Ac (pH 8.3) prior to analysis.

5.2.2 Dialysis

Samples of kidney cytosol were dialysed against 3x4 L of distilled deionized water (DDW) using Spectapor membrane tubings (Spectrum Medical Industries, Inc.) with a molecular weight cut-off (MWCO) of 3 500. The dialysis bags were washed before use as described in [70]. The dialysed as well as undialysed samples were evaporated to dryness under an IR lamp and the residues analyzed for their elemental content by INAA.

5.2.3 Fractionation by Precipitation with Ammonium Sulfate

Many enzymes are known to precipitate from solution over a narrow range of salt concentrations. Salt precipitation is therefore widely used as an initial step in protein purification. The one-step purification of glyceraldehyde phosphate dehydrogenase [186] is an interesting example where ammonium sulfate precipitation alone was applied to achieve sufficient purification of the enzyme.

The possibility of fractionation of metal-bound proteins from the cytosol fraction by ammonium sulfate precipitation was investigated. A cytosol sample of 80 mL was treated with solid $(NH_4)_2SO_4$ (Fisher Scientific) to give a final concentration of 1 M of the salt (26.6% saturation). The precipitate was collected by centrifugation at 5 000xg for 15 min. The resulting supernatant was then made 2 M (53.3% saturation), 3 M (79.9% saturation), and 4 M (100% saturation) with

respect to the salt and each time the precipitate was collected by centrifugation. All precipitates were dissolved in water. Resulting solutions and the final supernatant were dialysed against DDW, analyzed for protein content using the Lowry method and for the elemental content by INAA after freeze-drying.

5.2.4 Ion Exchange Chromatography

5.2.4.1 Selection of Salt for Ion Exchange Chromatography

In this ion-exchange chromatography experiment, an attempt was made to completely eliminate NaCl as the eluting salt. In INAA, it is extremaly difficult to analyze samples containing high levels of NaCl without a prior dialysis. There is always a possibility of losing some material during the transfer of the sample several times between test tubes, dialysis bags and irradiation vials, *etc.* This step could be eliminated if a salt was used where its matix did not get highly activated in NAA.

The number of salts which do not contain any elements that could interfere in INAA is limited. The following salts were considered for making up an eluting gradient: (i) lithium nitrate, $LiNO_3$; (ii) lithium phosphate, monobasic, LiH_2PO_4; and (iii) ammonium acetate, $CH_3CO_2NH_4$.

5.2.4.2 Ion Exchange Chromatography Experiments

A sample of 300 mL of cytosol was concentrated 3-fold using Aquacide and then dialysed against 0.1 M Tris/Acetate buffer (pH 8.3) before applying to the

DEAE-Sepharose column. A flow rate of 0.75 mL min^{-1} was selected and fractions of 6 mL were collected. Proteins were eluted from the column by a two-step salt gradient of ammonium acetate, *viz.* 0 - 0.5 M and 0.5 M - 1 M. Fractions were collected directly in medium-size irradiation vials and dried under an IR lamp prior to multielement analysis by INAA.

5.3 RESULTS AND DISCUSSION

5.3.1 Dialysis

Elemental content of the cytosol fraction before and after dialysis is presented in Table 5.1. Since the MWCO of the dialysis tubing was 3 500, elements detected in a dialysed sample can be considered as attached to macromolecules. More than 90% of Br, Cl, F, K, Na and Rb dialysed off; they must therefore exist predominantly as free ions or small molecules. Calcium, Mg and Mn dialysed in 35, 24, and 22%, respectively. It was concluded that most of Cu, Fe, Mo, S, Se, V and Zn in cytosol are bound to macromolecules as ≤ 10% of their total content passed through the dialysis membranes.

5.3.2 Fractionation by Precipitation with Ammonium Sulfate

Ammonium sulfate was analyzed for the presence of trace elemental impurities and the data are presented in Table 5.2. This reagent was found to be contaminated with high levels of Cr (0.21 ppm), Cu (0.76 ppm) and Zn (0.95 ppm). It is therefore possible that these elements may bind to proteins to certain extent

Table 5.1

Dialysability of elements present in bovine kidney cytosol[a]

Element	Undialysed Cytosol (ppm)	Dialysed Cytosol (ppm)	Dialysable (%)
Br	0.53 ± 0.03	0.048 ± 0.001	90.9 ± 7.9
Ca	20.9 ± 4.1	13.51 ± 2.75	35.4 ± 10.0
Cl	148.6 ± 5.2	2.54 ± 0.53	98.3 ± 20.8
Cu	0.34 ± 0.06	0.315 ± 0.028	7.3 ± 1.4
F	3.07 ± 0.94	0.17 ± 0.03	94.5 ± 33.4
Fe	6.12 ± 0.31	5.81 ± 0.74	5.1 ± 0.7
K	186.6 ± 6.7	9.29 ± 0.66	95.0 ± 7.5
Mg	21.58 ± 2.01	16.36 ± 0.18	24.2 ± 2.3
Mn	0.064 ± 0.007	0.05 ± 0.01	21.9 ± 5.0
Mo	0.037 ± 0.003	0.033 ± 0.002	10.8 ± 1.1
Na	129.2 ± 4.0	7.23 ± 0.6	94.4 ± 8.4
Rb	0.64 ± 0.10	0.042 ± 0.004	93.5 ± 17.1
S	631.0 ± 141.2	554.6 ± 60.3	12.1 ± 3.0
Se	0.029 ± 0.001	0.0266 ± 0.0006	8.3 ± 0.3
V	0.006 ± 0.001	0.0068 ± 0.0009	0
Zn	1.58 ± 0.29	1.48 ± 0.03	6.3 ± 1.2

[a] average of 6 determinations

Table 5.2

Elemental contaminants detected in ammonium sulfate[a]

Element	Concentration (ppm)
Br	0.031 ± 0.002
Cl	4.29 ± 042
Cu	0.76 ± 0.08
Cr	0.21 ± 0.02
K	1.88 ± 0.06
Mn	0.021 ± 0.003
Na	1.84 ± 0.31
Sb	0.072 ± 0.003
Zn	0.95 ± 0.09

[a] average of 7 determinations.

over the experimental time. This reagent is used in large quantities for protein precipitation and therefore caution must be taken while the levels of protein-bound Cr, Cu and Zn in biological materials are to be evaluated.

The percent recoveries of elements after the stepwise ammonium sulfate precipitation are shown in Table 5.3. The protein content of the fractions is presented in Table 5.4. Ratio of trace element present in a particular fraction to the protein content in that fraction is shown in Table 5.5. Any number larger than 1 in Table 5.5 is interpreted as indication of an enrichment over a starting material. Percent distribution of protein in final supernatant was very small, *viz.* 1.8%, and therefore even if present in very small amounts an element might still get enriched compared to its level in the original sample. Such was the case for Cu, Cr and Mn. On the other hand, the overall recovery of the element in this fraction was still very poor. Two of the elements mentioned above, *viz.* Cu and Cr, were detected as impurities in ammonium sulfate therefore their high content in the supernatant fraction most likely came from this reagent. Majority of the elements precipitated at 2- and 3-M salt levels as did most of the proteins. More than 50% of Cu, Fe, Mo, Se, V and Zn were detected in these two fractions, but only Cu, Se and V got enriched in the 2 M ppt. The 1 M salt can be used for precipitation of Ca, Cr, Fe, Mn, Mo, Se and Zn. Iron, Mg and V concentrated most in the 4 M ppt. Again, Cr, Cu and Zn results may be erroneous due to the presence of these elements as contaminants in the ammonium sulfate itself.

In conclusion, ammonium sulfate precipitation can be successfully applied

Table 5.3

Percent distribution of trace elements after ammonium sulfate fractionation[a]

Element	1 M ppt.	2 M ppt.	3 M ppt.	4 M ppt.	Final Supernatant	Total Recovery
Br	0.20 ± 0.01	2.2 ± 0.1	2.6 ± 0.2	2.10 ± 0.06	0.30 ± 0.02	7.4 ± 0.2
Ca	8.6 ± 0.6	7.3 ± 0.3	39.4 ± 3.5	4.3 ± 0.3	0.70 ± 0.06	60.3 ± 3.5
Cl	0.60 ± 0.02	1.30 ± 0.09	1.7 ± 0.1	1.30 ± 0.08	0.040 ± 0.003	4.8 ± 0.2
Cr[b]	18.7 ± 0.8	34.5 ± 2.8	25.7 ± 1.0	8.7 ± 0.9	12.4 ± 1.2	100 ± 3.4
Cu	4.4 ± 0.3	46.0 ± 4.1	33.0 ± 2.3	19.3 ± 1.7	4.6 ± 0.4	107.4 ± 5.0
F	N/D	N/D	3.5 ± 0.3	8.3 ± 0.7	N/D	11.8 ± 0.8
Fe	20.7 ± 1.7	25.0 ± 1.4	20.6 ± 1.4	30.7 ± 3.4	N/D	97.0 ± 4.3
K	1.00 ± 0.06	1.30 ± 0.08	1.40 ± 0.08	1.2 ± 0.1	1.20 ± 0.10	6.1 ± 0.2
Mg	0.50 ± 0.05	8.2 ± 0.7	23.7 ± 1.2	26.7 ± 3.5	0.40 ± 0.04	59.5 ± 3.8

Table 5.3

Percent distribution of trace elements after ammonium sulfate fractionation (continued)

Element	1 M ppt.	2 M ppt.	3 M ppt.	4 M ppt.	Final Supernatant	Total Recovery
Mn	8.4 ± 0.8	19.8 ± 1.2	17.9 ± 1.6	20.6 ± 1.6	15.3 ± 1.1	82.1 ± 2.9
Mo	25 ± 2.4	41.9 ± 3.8	16.2 ± 1.6	10.8 ± 0.5	N/D	93.9 ± 4.8
Na	2.3 ± 0.2	1.20 ± 0.05	1.20 ± 0.07	1.00 ± 0.03	1.90 ± 0.15	7.6 ± 0.3
Rb	1.20 ± 0.07	N/D	5.4 ± 0.5	2.6 ± 0.2	N/D	9.3 ± 0.5
Se	5.8 ± 0.4	55.4 ± 5.0	26.3 ± 1.1	6.7 ± 0.5	0.30 ± 0.02	94.2 ± 5.2
V	13.2 ± 0.8	50.4 ± 6.0	6.4 ± 0.2	25.6 ± 3.1	N/D	95.6 ± 6.8
Zn	8.2 ± 0.7	42.8 ± 4.3	25.3 ± 1.8	10.5 ± 0.5	0.20 ± 0.01	87.1 ± 4.7

[a] calculated per fresh sample;

[b] assuming 100% recovery;

N/D = not detected.

Table 5.4

Protein content of ammonium sulfate precipitates[a]

Fraction	Concentration (mg mL^{-1})	Total Content (mg)	Distribution (%)
Initial Cytosol	3.41 ± 0.11	272.8 ± 8.8	(100)
1 M precipitate	3.18 ± 0.16	12.72 ± 0.64	4.7 ± 0.3
2 M precipitate	11.31 ± 0.74	101.8 ± 6.6	37.3 ± 2.8
3 M precipitate	9.86 ± 0.69	88.7 ± 6.2	32.5 ± 2.1
4 M precipitate	4.92 ± 0.39	47.72 ± 3.82	17.5 ± 1.6
Final Supernatant	0.046 ± 0.002	4.88 ± 0.24	1.8 ± 0.1

[a] average of 7 determinations

Table 5.5

Ratio of trace element content to protein content in ammonium sulfate precipitates[a]

Element	1 M ppt.	2 M ppt.	3 M ppt.	4 M ppt.	Final Supernatant
Br	0.040 ± 0.004	0.060 ± 0.004	0.080 ± 0.007	0.120 ± 0.003	0.17 ± 0.02
Ca	1.83 ± 0.20	0.20 ± 0.01	1.21 ± 0.12	0.25 ± 0.02	0.38 ± 0.05
Cl	0.13 ± 0.01	0.030 ± 0.003	0.050 ± 0.004	0.070 ± 0.004	0.020 ± 0.002
Cr	3.98 ± 0.38	0.92 ± 0.09	0.79 ± 0.05	0.50 ± 0.05	6.89 ± 0.94
Cu	0.94 ± 0.10	1.23 ± 0.01	1.01 ± 0.09	1.10 ± 0.10	2.56 ± 0.33
F	N/D	N/D	0.11 ± 0.01	0.47 ± 0.04	N/D
Fe	4.40 ± 0.52	0.67 ± 0.05	0.63 ± 0.05	1.75 ± 0.19	N/D

Table 5.5. Ratio of trace element content to protein content in ammonium sulfate precipitates (continued)

Element	1 M ppt.	2 M ppt.	3 M ppt.	4 M ppt.	Final Supernatant
K	0.21 ± 0.02	0.03 ± 0.12	0.040 ± 0.003	0.070 ± 0.006	0.67 ± 0.09
Mg	0.11 ± 0.01	0.22 ± 0.02	0.73 ± 0.05	1.53 ± 0.20	0.22 ± 0.03
Mn	1.79 ± 0.23	0.53 ± 0.04	0.55 ± 0.06	1.18 ± 0.09	8.50 ± 1.02
Mo	5.32 ± 0.02	1.12 ± 0.01	0.50 ± 0.06	0.62 ± 0.03	N/D
Na	0.49 ± 0.06	0.030 ± 0.002	0.040 ± 0.003	0.060 ± 0.002	1.06 ± 0.13
Rb	0.26 ± 0.03	N/D	0.17 ± 0.02	0.15 ± 0.01	N/D
Se	1.23 ± 0.13	1.49 ± 0.16	0.81 ± 0.05	0.37 ±0.03	0.17 ± 0.02
V	2.81 ± 0.29	1.35 ± 0.18	0.20 ± 0.01	1.46 ± 0.18	N/D
Zn	1.74 ± 0.21	1.15 ± 0.13	0.78 ± 0.07	0.60 ± 0.03	0.11 ± 0.01

[a] average of 5 determinations;

N/D = not detected.

as an initial step for the fractionation of protein-bound trace elements. Various amounts of elements were detected in the salt precipitates and the calculated enrichment fractor may indicate the most advantegous salt concentration for the purification of an element of interest.

5.3.3 Ion Exchange Chromatography

Inorganic impurities present in Aquacide, Tris and the DEAE-Sepharose are shown in Table 5.6; only low levels of Br, Cl and Na were detected. These elements exist in the aqueous solutions as monovalent ions and hence did not interfere with the analysis of trace elements bound to macromolecules.

Table 5.6

Elemental impurities detected Tris and DEAE-Sepharose[a]

Element	Concentration (ppm)	
	Tris	DEAE-Sepharose
Br	0.10 ± 0.02	0.52 ± 0.04
Cl	< 0.55	11.8 ± 0.4
Na	< 0.58	10.8 ± 0.3

[a] average of 8 determinations.

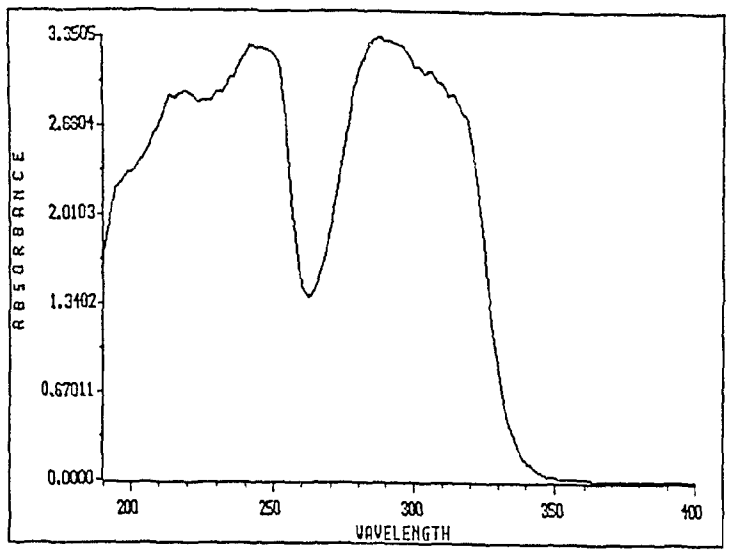
As mentioned earlier, lithium nitrate, lithium phosphate and ammonium acetate were considered for the elution of proteins.

Proteins are detected in the chromatographic experiments by measuring absorbance at 280 nm. As the nitro-group also absorbs light with a maximum absorbance at exactly the same wavelength [187], lithium nitrate solution could not be used in conjunction with protein detection. Examples of the absorbance measurements of 1 M $LiNO_3$ and protein (bovine serum albumin) using a quartz photo cell QS are presented in Fig. 5.1(a) and 5.1(b), respectively. Increased salt concentration would cause an increase in the absorbance and mask completely any protein present in a sample.

The attempts to use LiH_2PO_4 were also unsuccessful. In order to prepare lithium phosphate solution, 1 M H_3PO_4 was titrated with lithium hydroxide to the desired pH. Addition of the base caused the formation of a precipitate that would dissolve at the beginning of titration but become insoluble upon further addition of the base. The CRC Handbook of Chemistry and Physics [188] does not provide any information on the solubility of monobasic lithium phosphate in water.

In the next step, 1 M ammonium acetate in Tris buffer was prepared from the solid salt. Preliminary experiments to compare the eluting abilities of this solution with a solution of NaCl were performed. A cytosol sample was analyzed twice by DEAE-Sepharose chromatography. In the first run, 0 - 1 M NaCl gradient was applied and in the second run 0 - 1 M $CH_3CO_2NH_4$ gradient was used. The chromatograms are presented in Figs. 5.2 and 5.3, respectively. No significant

(a)



(b)

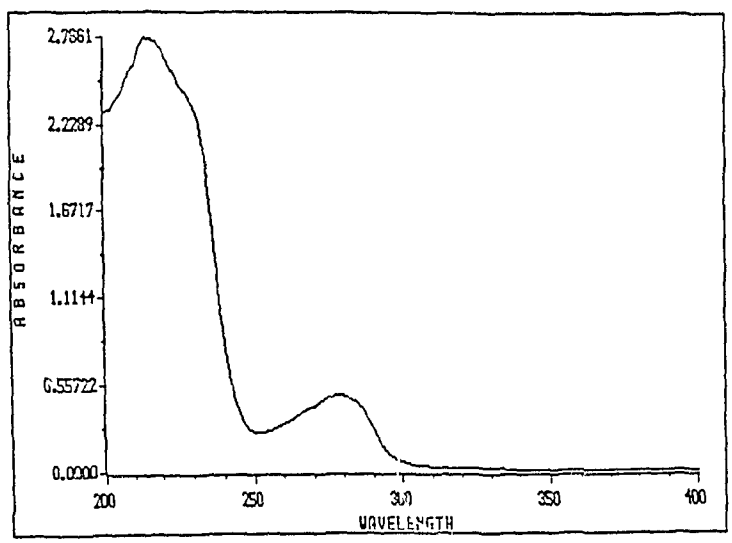


Fig. 5.1. Absorbance spectrum of: (a) lithium nitrate, (b) bovine serum albumin. Wavelength range:190 to 400 nm.

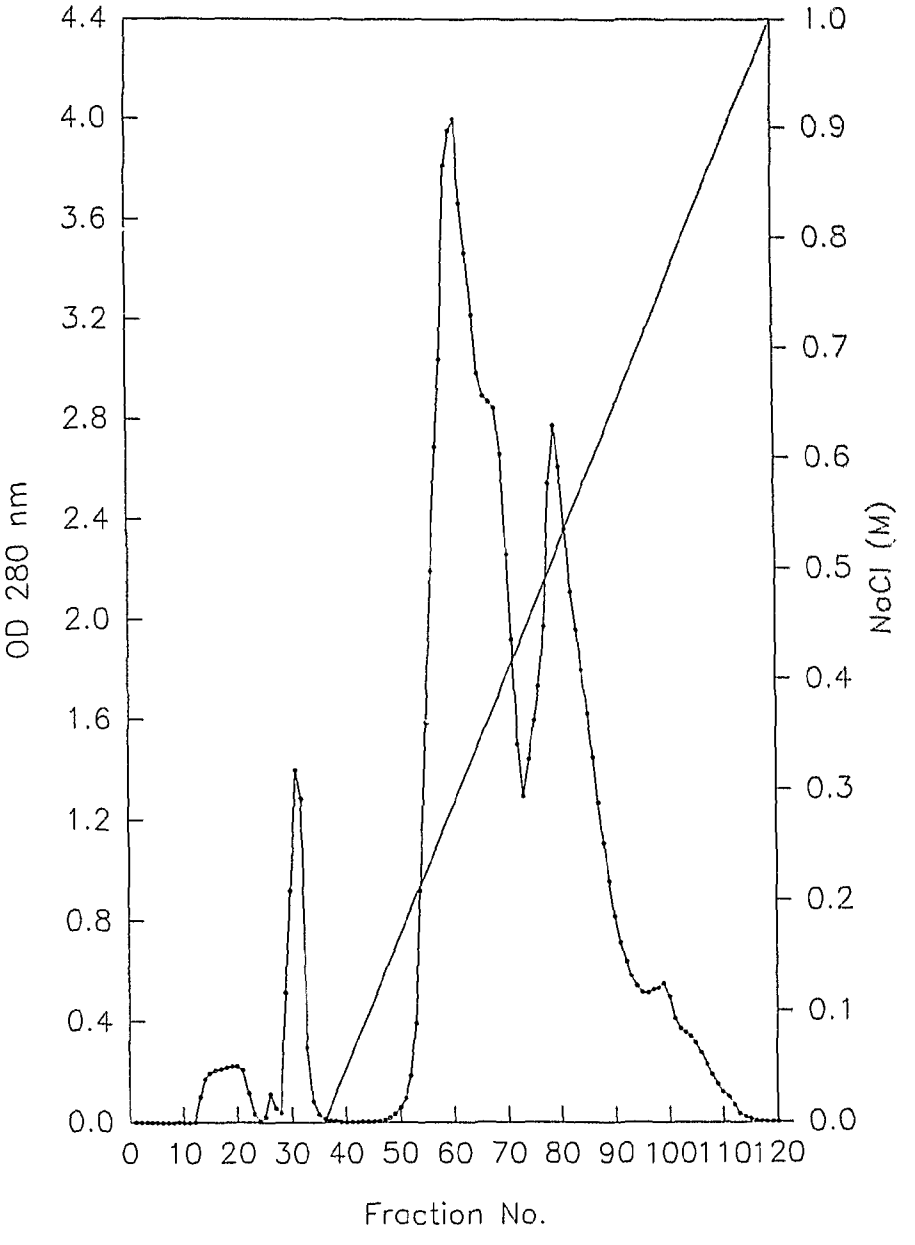


Fig. 5.2. Elution profile of cytosol on a DEAE-Sepharose column using NaCl as the eluting medium.

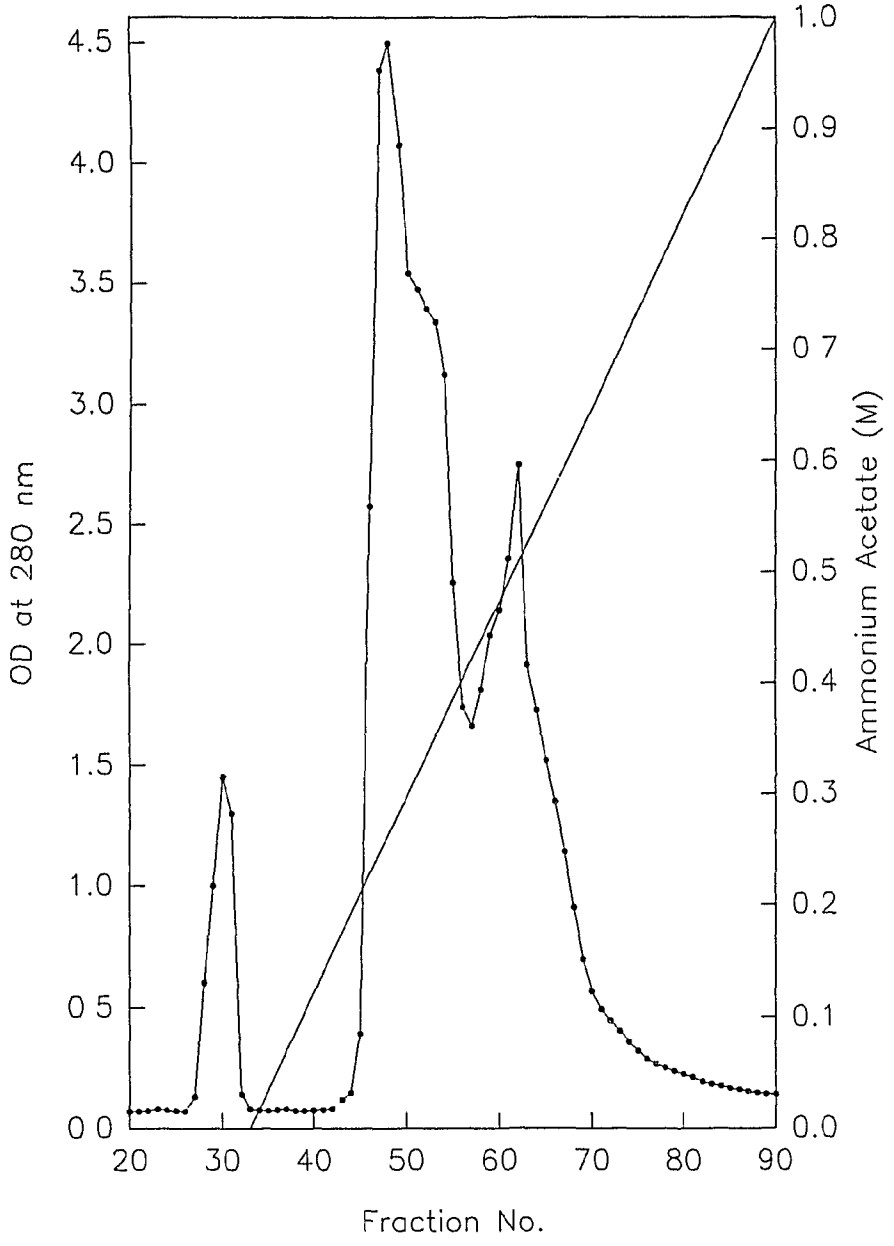


Fig. 5.3. Elution profile of cytosol on a DEAE-Sepharose column using $CH_3CO_2NH_4$ as the eluting medium.

difference was noticed in terms of the elution pattern of the proteins in both experiments. Ammonium acetate was therefore selected for a salt gradient.

Elemental impurities present in solid ammonium acetate are shown in Table 5.7. Only very low levels of Br, I and Na were detected. These amounts do not interfere in the determination of other trace elements by NAA.

Table 5.7

Elemental impurities detected in ammonium actetate[a]

Element	Concentration (ppm)
Br	0.085 ± 0.006
I	0.065 ± 0.003
Na	2.56 ± 0.35

[a] average of 8 determinations.

A sample of cytosol was subjected to ion exchange chromatography as described in Section 5.2.4.2. Seven protein-bound elements were well resolved on a DEAE-Sepharose column. The elements were: As, Cu, I, Mn, Se, V and Zn. Selenium has already been discussed in detail in Chapter 4. The results for the other six elements are presented below in separate sections.

5.4 ARSENIC

The signs of arsenic deprivation have been observed in four animal species, *viz.* chick, goat, miniature pig and rat [189], with symptoms such as depressed growth and abnormal reproduction. However, all observations of As essentiality come from animal studies and simple extrapolation to humans cannot easily be performed. A plausible essentiality of As for man was suggested by Mayer *et al.* [190] who studied As levels in patients with renal failure undergoing haemodialysis treatment. A significantly lower As concentration was found in blood serum of those persons compared to the healthy controls and this As deficiency could contribute to the increased death risk of the affected people.

To date, however, As is well-known for its toxicity. According to the International Agency for Research on Cancer (IARC) [113], As and its inorganic compounds are carcinogenic to humans. Exposure to high levels of As and its salts might cause skin [113], [191], [192] or lung cancer [113]. On the other hand, organic arsenic compound arsenobetaine which is the main form of As in certain seafoods was found to be non-toxic and non-carcinogenic by Sabbioni *et al.* [193].

Arsenic is present in all living materials but its most important chemical form in nutrition and physiology of living organisms is still not known. Studies on As distribution performed by Neiger and Osweiler [194] on dogs fed low-level As diet showed that As concentrations in tissues and body fluids reflected As levels in the diet and that urine and hair could be the best specimens to analyze for monitoring As exposure or poisoning. Pederson *et al.* [195] reported that As accumulated in

rats to a greater extent in blood followed by kidney, hair and liver. Within subcellular fractions of liver it accumulated most in the nuclei followed by cytosol, mitochondria and microsomes. In bovine kidney studied by Jayawickreme [70], the cytosol fraction was found to contain the highest amount of As followed by nuclei, mitochondria, lysosomes and microsomes.

Metabolism of As in rats and rabbits was investigated by Marafante *et al.* [196] using ^{74}As radiotracer. Different species of As in urine were detected including inorganic As, monomethylated As and dimethyl-arsinic acid. This study confirmed the proposed involvement of methylated groups in the excretion process of As from the body.

Although the amount of total As in different biological specimens is frequently reported, little information is available on As bound to macromolecules. Arsenic in bovine kidney supernatant fraction was studied by Jayawickreme [70] using various bioanalytical techniques followed by INAA. Behavior of As during ammonium sulfate precipitation, gel filtration, ion exchange chromatography, isoelectric focusing, chromatofocusing and the stability of As-protein complexes were investigated. The presence of one major As-binding protein was reported; it had a MW of approximately 38 kDa and a pI of 4.9.

In the present study, As could not be detected in most cases due to either too low As levels in the samples analyzed or spectral interferences from the adjacent 554-keV photopeak of ^{82}Br towards the 559-keV photopeak of ^{76}As. Arsenic could not be detected in whole kidney and its subcellular fractions and in

ammonium sulfate precipitates. However, it was possible to detect As in cytosol after ion exchange chromatography, most likely due to the separation of the majority of monovalent and interferring ions such as Br^-, Na^+, and Cl^- from the As activity. The elution profile of proteins and As on a DEAE-Sepharose column is shown in Fig. 5.4. Arsenic fractionated into two peaks which eluted close to each other by 0.25 M and 0.3 M ammonium acetate, respectively. About 0.175 µg of As eluted from the column. No other information can be provided at this point because As could not be detected in samples applied to the column.

These results differ from those obtained by Jayawickreme [70]. He separated As into two peaks, one which eluted without binding to the column and the other one by 0.1 M NaCl. However, his studies were done on the kidney supernatant, prepared by centrifugation at 30 000xg for 25 min. Consequently, his samples contained microsomal and cytosolic fractions together. It is therefore possible that the first As peak might have come from the microsomal proteins. The difference in the elution of As-binding proteins cannot be explained at this point. The use of different ion-exchange gels and eluting media in both experiments might account for these disagreements. Further studies are needed.

5.5 IODINE

Human body contains about 10-20 mg of iodine, 80% of which is concentrated in the thyroid gland. Other body pools of iodine include ovaries, skeletal muscles, salivary glands and the obitary fat and the obicular muscle of

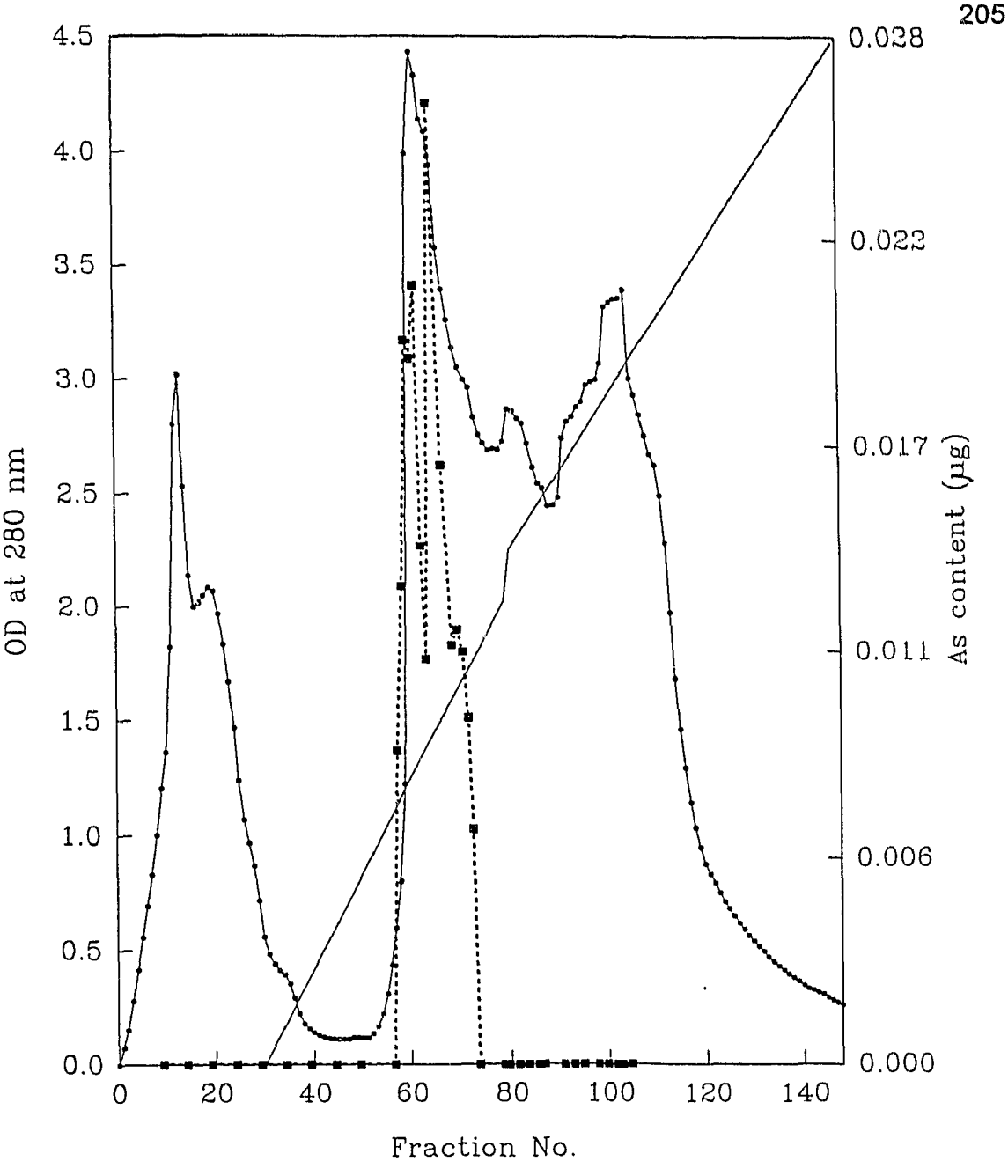


Fig. 5.4. Elution profile of proteins and arsenic in cytosol on a DEAE-Sepharose column. Protein: (------), arsenic: (■■■).

eye. The thyroid gland normally contains about 10 mg of iodine and 90% of it is covalently linked to thyroglobulin, a glycoprotein with MW of 660 kDa. In blood, iodine exists in both inorganic and organic forms. Inorganic iodine is thought not to be bound and it is cleared principally by the thyroid gland and kidneys. Almost all of the organic iodine is present as thyroxine bound to the plasma proteins [197].

Iodine deficiency in diet is the most common cause of goitre and hypothyroidism and an excess of the element may lead to hyperthyroidism. In humans and animals, the thyroid hormone is essential for growth during early years, therefore monitoring the status of iodine in infants and children is of great importance. Fetal iodine deficiency, an endemic condition affecting the development of brain, occurs in man in association with endemic goitre and severe dietary iodine deficiency. This disease and other brain retardation disorders have been studied by Hetzel [198] and Potter *et al.* [199].

Iodine can easily pass the mammary barrier and its level in milk has been proposed as an useful index of variations in dietary iodine intakes [200] and of the iodine status of goitrous regions [42].

Severe iodine toxicity in man may take place *via* an exposure to the radioactive ^{131}I after a nuclear power plant accident or from fall out of nuclear explosion [201]. The isotope is mainly incorporated in the body *via* food. Potassium iodide ($K^{127}I$) is used as an antidote to block uptake of radioactive iodine by the thyroid gland in such cases [197].

Iodine in biological materials is often determined using NAA but application

of a preconcentration or separation method is needed to eliminate interferences from the high activities of Na, K, Br and Cl and to measure ppb levels of iodine. Methods for the determination of ng levels of iodine in foods and biological samples using radiochemical NAA [202], and microwave acid digestion and preconcentration NAA [203] have been developed by Rao and Chatt.

Recent discovery that the enzyme iodothyronine deiodinase contains selenocysteine [37] relates Se with some essential functions of iodine. Studies on the effect of Se deficiency and supplementation on thyroid hormone metabolism have been reported [204], [205].

Information on the distribution of iodine in different subcellular fractions of animal organs is limited. Jayawickreme [70] studied bovine kidneys and found that about 50% of iodine was present in the cytosol fraction. Application of gel filtration, isoelectric focusing and chromatofocusing to the supernatant fraction demonstrated the presence of at least one iodine-binding protein. The MW of this protein and its pI were determined as 71 kDa and 4.5, respectively.

In the present work, it was rather impossibile to detect very low concentrations of iodine in the samples studied using conventional INAA. The only positive experiment that can be briefly discussed is ion exchange chromatography on a DEAE-Sepharose column. The elution profile of proteins and iodine from the cytosol fraction is presented in Fig. 5.5. Iodine was detected in at least 6 peaks. The first peak was associated with the material that did not bind to the column and the rest eluted at various concentrations of ammonium acetate, ranging from 0.3 M

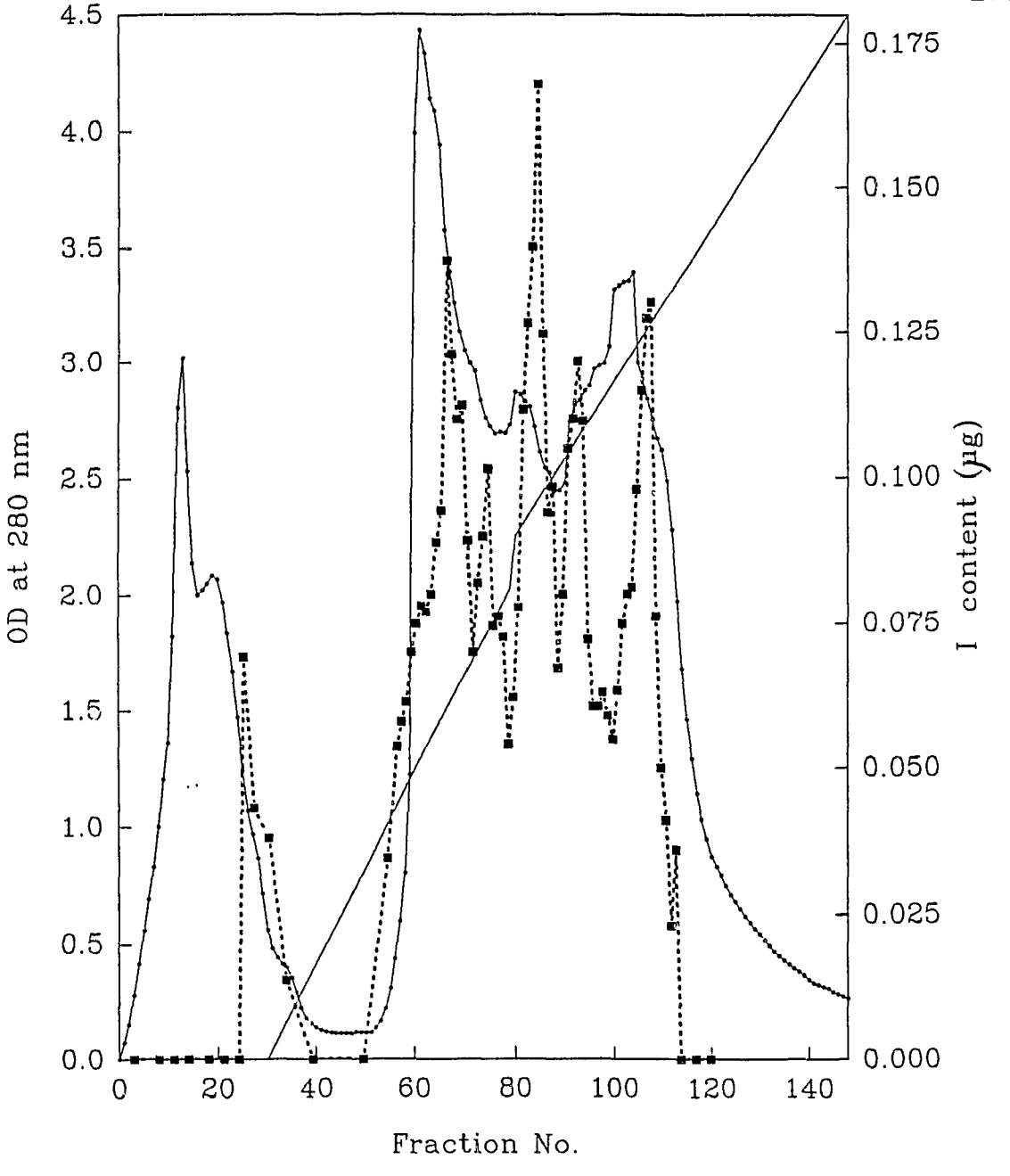


Fig. 5.5. Elution profile of proteins and iodine in cytosol on a DEAE-Sepharose column. Protein: (∿∿∿∿), iodine: (-■-■-■-).

to 0.7 M. These findings are different from what was reported previously from our laboratory by Jayawickreme [70], where only two iodine-protein complexes separated on the column. Not enough information is provided at this point to make a comparison and to draw any constructive conclusions. The INAA and biochemical methods used in this study were specifically developed for the determination of Se in biological samples and the procedures lacked sensitivity for iodine determination in the cytosol fraction.

5.6 VANADIUM

Although research on V has been of great interest for some time, its biochemical and physiological role has not been fully established. Evidence for the essentiality of vanadium for chicks and rats was reported by several different research groups in the early 1970s. Vanadium deficiency resulted in impaired bone development, reduced growth and disturbances in blood metabolism [197]. Further studies suggested that V might be involved in maintaining serum glucose level, in iodine metabolism and in thyroid functions [189]. It has also been postulated that V might play a role in the regulation of Na,K-ATPase and H,K-ATPase in renal cells. Sitprija *et al.* [206] studied metabolic problems in people in Thailand who developed decreased activity of Na,K-ATPase and H,K-ATPase. High levels of V were found in the soil and also in urine, kidneys and lungs of the villagers. It was suggestedd that V was responsible for the existing health problems.

Since mid 1980s, extensive studies have been carried out in order to examine the mechanism through which V compounds, mainly vanadate, mimics the action of insulin in preventing the signs of diabetes in rats [197]. Tissue V content in normal and diabetic rats was measured by Hamel *et al.* [207]. Several organs were investigated and V content in liver was significantly lower in diabetic animals. These findings suggested the importance of V in insulin homeostasis. Domingo *et al.* [208], [209] and Oster *et al.* [210] investigated the effect of oral V administration on diabetic rats. Although glucose homeostasis improved and some signs of diabetes ameliorated, but at the same time negative side effects were observed in all of the V-treated animals. These effects included deaths, decreased weight gain and tissue V accumulation. Much more extrensive research needs to be done in order to apply V compounds as an alternative agent to insulin in humans.

Vanadium is toxic if taken at high levels. Toxicity of V has been induced in laboratory animals [197]. Toxic effects of V on human respiratory system were demonstrated in workers exposed to V-containing dust and fumes [211]. Presently, no data exist indicating that V is carcinogenic to animals or man [113].

Vanadium levels in humans and animal tissues are very low, typically in the ppb range; the highest levels are detected in liver, kidney and bone [212], [213]. Human liver and blood were analyzed by Cornelis *et al.* [214] using RNAA. Liver V content ranged from 2.53 to 13.4 ng per g of fresh tissue, while blood V levels were extremely low, *e.g.* between 17 and 66 pg mL^{-1}. Hopkins and Tilton [215]

investigated subcellular distribution of V in rat liver using ^{48}V. Shortly after injection, the majority of the element was found in cytosol but with time the radioactivity shifted from this fraction into nuclei and mitochondria. Similar studies done by Sharma *et al.* [216] reached different conclusions. The element was found to be evenly distributed among all fractions and within the supernatant it was associated with high-MW proteins. Studies on rat [217] and human [90] blood were done by Sabbioni and Marafante. Vanadium was found to be associated with transferrin, the Fe-transport protein. Transferrin delivers Fe to the bone marrow and it was suggested by the authors that V might also be carried there along with Fe. This could explain the retention of V and its high content in bones and teeth as reported by several researchers [212], [213], [42].

Vanadium in bovine kidneys was studied by Jayawickreme using INAA [70]. Generally, low levels of the element and contamination from reagents did not allow a very thorough investigation. The highest V content was found in cytosol, followed by nuclei and mitochondria. No V was found in dialysates of the subcellular fractions. The highest elemental content was found in the 2 M ppt. in ammonium sulfate precipitation. The V-containing proteins did not bind to ion exchange chromatography column.

Vanadium was found to be an essential element for bacteria, algae and fungi. Bergstrom et al. [218], Eady *et al.* [219] and Miller and Eady [220] investigated V-containing nitrogenases from *Azotobacter chroococcum.* Other enzymes discovered in algae include iodoperoxidases and bromoperoxidases [2].

A novel V enzyme, chloroperoxidase, purified from the fungus *Curvularia inaequalis* was reported by van Schijndel *et al.* [221]. No V-dependent protein has yet been found in animals and man.

In the present study, V concentration in the bovine kidney homogenate was 81 ± 15.0 ppb as shown in Table 5.8. This value is higher compared to the reported values of 49.05 ± 2.49 ppb [213], 41.4 ± 4.2 ppb [207] in rat kidneys and 37 ± 8 ppb [70] in bovine kidneys. No V contamination was found in sucrose and HEPES used for buffer preparation and corrections were made for the presence of V in polyethylene irradiation vials. No other detectable source of erroneous results was present in the studies. The highest amount of V was found in the nuclei fraction and this is in a good agreement with majority of the reported data. Nuclei contained 58.8% of the element followed by mitochondria (34.5%), lysosomes (26.1%) and microsomes (6.6%). The level of V was below detection limit in the cytosol fraction. However, its concentration could be determined in the dialysed cytosol sample and was found to be 6.8 ± 0.9 ppb (Table 5.1). It was reported from our laboratory in the past [70] that V did not dialyse from cytosol. The concentraiton of 6.8 ppb could therefore refer to the concentration of V in the undialysed cytosol fraction.

Table 5.8

Vanadium in homogenate and in subcellular fractions of bovine kidneys

Subcellular Fraction	Concentration (ppb)[a]	Distribution (%)
Homogenate	81.0 ± 15.0	(100)
Nuclei	48.0 ± 15.0	58.8 ± 6.1
Mitochondria	28.0 ± 5.0	34.5 ± 6.0
Lysosomes	21.0 ± 9.0	26.1 ± 11.2
Microsomes	5.0 ± 2.0	6.6 ± 2.5
Cytosol	< 7.0	N/D

[a] expressed as ng per g of wet sample;

N/D = not detected.

The fraction obtained by precipitation with 2 M ammonium sulfate (Table 5.3) accounted for 50.4% of the total V. The second richest fraction was the 4 M ppt. containing 25.6% of total V. However, V-to-protein ratio (2.81, Table 5.5) showed the highest enrichment of V in the 1 M ppt. The results therefore indicate the presence of two or three V-containing components in cytosol. The 1 M ammonium sulfate could be successively applied as an initial step for the purification of V-containing proteins. The overall recovery of the element was 95.6%.

Fractionation of V-containing proteins on a DEAE-Sepharose column was

investigated and the elution profile is presented in Fig.5.6. Insignificant amounts of V eluted without binding to the column. On the column, V separated on one major and two minor peaks. The first peak was the largest one and eluted by 0.3 M ammonium acetate; the second peak was smaller and eluted by 0.5 M salt; the third peak was the smallest and eluted by 0.63 M salt. The first peak had a V concentration twice of that of the initial sample. The overall recovery was 77%. In gel filtration experiment of the supernatant fraction of rat liver, Sharma *et al.* [216] reported the elution of V along with high-MW proteins. However, no information is available regarding V-containing proteins in the cytosol fraction of bovine kidneys. Kidneys were found to contain the highest amounts of V among all organs, yet no physiological function of V has been proposed so far.

5.7 MANGANESE

Manganese was shown to be essential for growth and reproduction of rats and mice and it prevented a skeletal abnormality in chickens [42]. No well defined case of Mn deficiency in man has yet been reported (with one exception where a volunteer received an experimental diet deficient in Mn and vitamin K [222]). On the other hand, Mn toxicity in humans has been widely reported as resulting from either acute or chronic exposure which resulted from occupational inhalation of Mn-containing particles or fumes, or from ingestion [223], [197].

In living organisms, Mn can function both as (i) a component of a metalloenzyme where the element has a structural or functional role; and (ii) as an

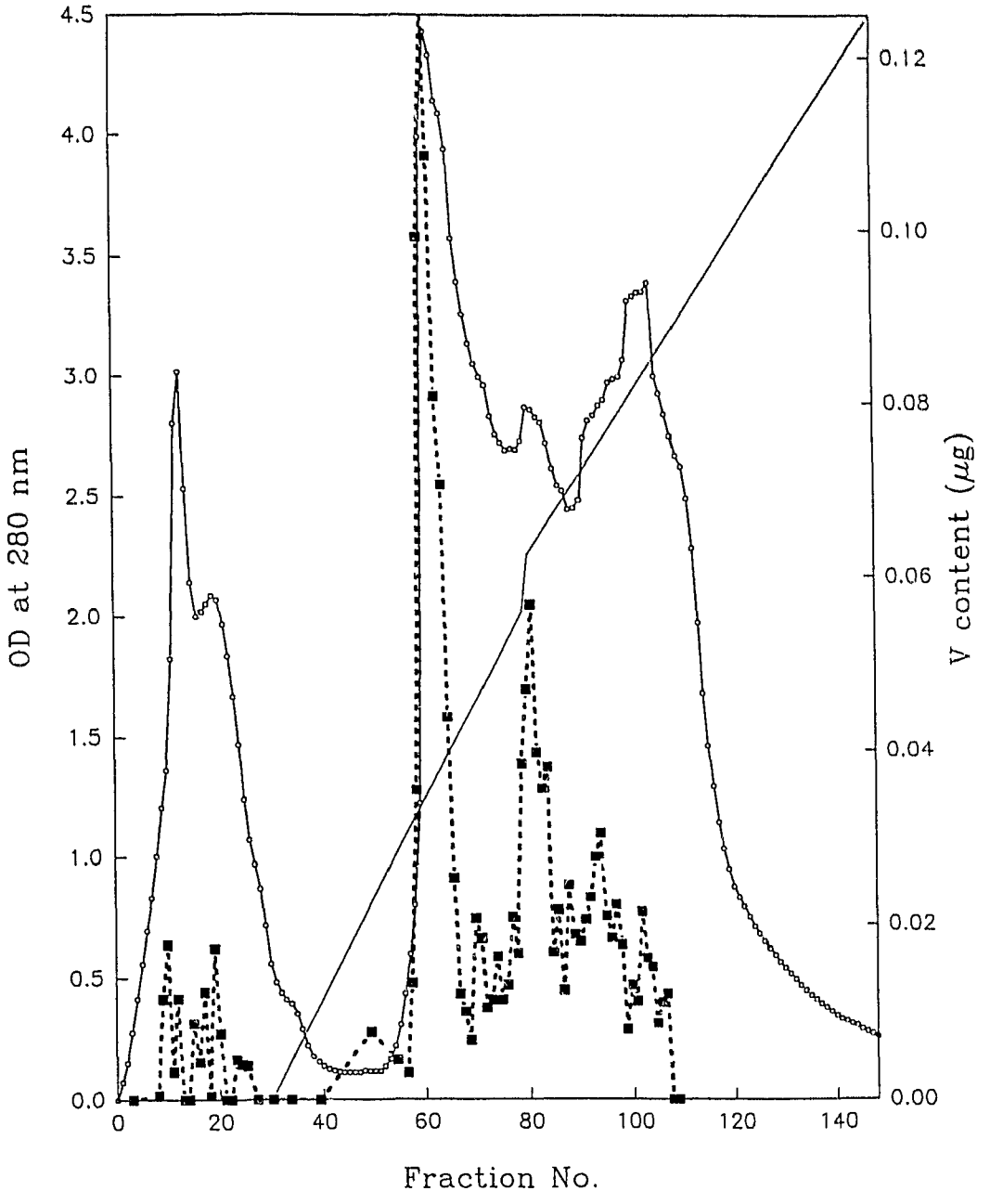


Fig. 5.6. Elution profile of proteins and vanadium in cytosol on a DEAE-Sepharose column. Protein: (------), vanadium: (■■■■).

enzyme activator where the element exists as a dissociable cofactor. To date, the following Mn-containing enzymes and proteins have been recognized: arginase, Mn-dependent superoxide dismutase, pseudocatalase, the catalytic subunit of calmodulin-dependent protein phosphatase, phytohemagglutinins, pyruvate carboxylase, α-isopropyl malate synthetase and the oxygen-evolving center of photosynthesis [223]. Enzymes activated by Mn^{2+} ion include kinases, decarboxylases, hydrolases and transferases. Majority of Mn-dependent proteins were isolated from tissues rich in mitochondria as Mn was found to be most abundant in this fraction [197].

Manganese is involved in carbohydrate metabolism, mainly in insulin metabolism, and in maintaining the tolerance for glucose. It is also essential for normal brain functions. Human epileptics have been reported to contain low blood Mn levels in comparison to healthy subjects [224].

A relationship between Fe and Mn metabolism have been observed in laboratory animals [42], [225]. The absorption and utilization of Fe are affected negatively by excessive dietary Mn, especially in cases of inadequate intake of Fe. Furthermore, Hartman *et al.* [226] observed that tissue Fe concentrations in lambs are reduced by high levels of Mn. The influence of Fe on Mn absorption in rats was studied by Davis *et al.* [227]. High Fe intakes inhibited Mn absorption, reduced tissue Mn concentration and inhibited Mn-dependent superoxide dismutase activity. These finding suggested that the Mn-to-Fe dietary ratio is of great significance in homeostasis of these elements.

Manganese-binding proteins in bovine kidney and its supernatant fraction were investigated by Jayawickreme [70]. Majority of Mn was detected in cytosol. Seven Mn peaks in supernatant were detected after ion exchange chromatography. On a gel filtration column, Mn-containing proteins separated into three major fractions with approximate molecular weights of 270 kDa, 89 kDa and 18 kDa, respectively. Problems with contamination coming from reagents were reported.

Manganese concentrations in bovine kidney homogenate and its subcellular fractions are given in Table 5.9. The value of 0.66 ± 0.08 ppm Mn in the homogenate agrees well with 0.72 ± 0.05 ppm reported for rat kidney [197]. According to the same authors, the concentration of Mn in various tissues is quite similar in most species. The element was found to be equally distributed between nuclei (30.6%), mitochondria (29.4%) and cytosol (25.9%). Only 7.4% and 5.9% of total Mn was detected in lysosmes and microsomes, respectively. During the dialysis of cytosol, 21.9% of Mn passed through a membrane with MWCO of 3500 and the remaining part was assumed to be protein-bound (Table 5.1).

The results of ammonium sulfate precipitation are presented in Tables 5.3 and 5.5. Total recovery of Mn was 82%. Fractions obtained by precipitation with 2-, 3-, and 4-M salt as well as the final supernatant contained almost equal amounts of Mn, ranging from 15.3% in the final supernatant to 20.6% in the 4 M ppt. The least amount of Mn (8.4%) was found in the 1 M ppt. Because both the 1 M ppt. and final supernatant contained the lowest levels of protein, the

Table 5.9

Manganese in homogenate and in subcellular fractions of bovine kidneys

Subcellular Fraction	Concentration (ppm)[a]	Distribution (%)
Homogenate	0.66 ± 0.08	(100)
Nuclei	0.20 ± 0.01	30.6 ± 2.1
Mitochondria	0.194 ± 0.009	29.4 ± 1.4
Lysosomes	0.05 ± 0.02	7.4 ± 3.3
Microsomes	0.039 ± 0.005	5.9 ± 0.7
Cytosol	0.17 ± 0.02	25.9 ± 3.7

[a] expressed as μg per g of wet sample.

Mn-to-protein ratios were highest in these two fractions: 1.79 for the 1 M ppt and as high as 8.5 in the final supernatant. Ammonium sulfate was found to contain 0.021 ± 0.003 ppm of Mn (Table 5.2). Although this level was one order of magnitude lower than that measured in the starting cytosol, this reagent blank contamination may still account for such high enrichment factor of Mn in the supernatant. The results obtained agree well with those reported by Jayawickreme [70].

The fractionation pattern of Mn-containing proteins on a DEAE-Sepharose column is shown in Fig. 5.7. Manganese eluted to a small extent as unbound material and on the column it separated into two major peaks at 0.3 M and 0.5 M

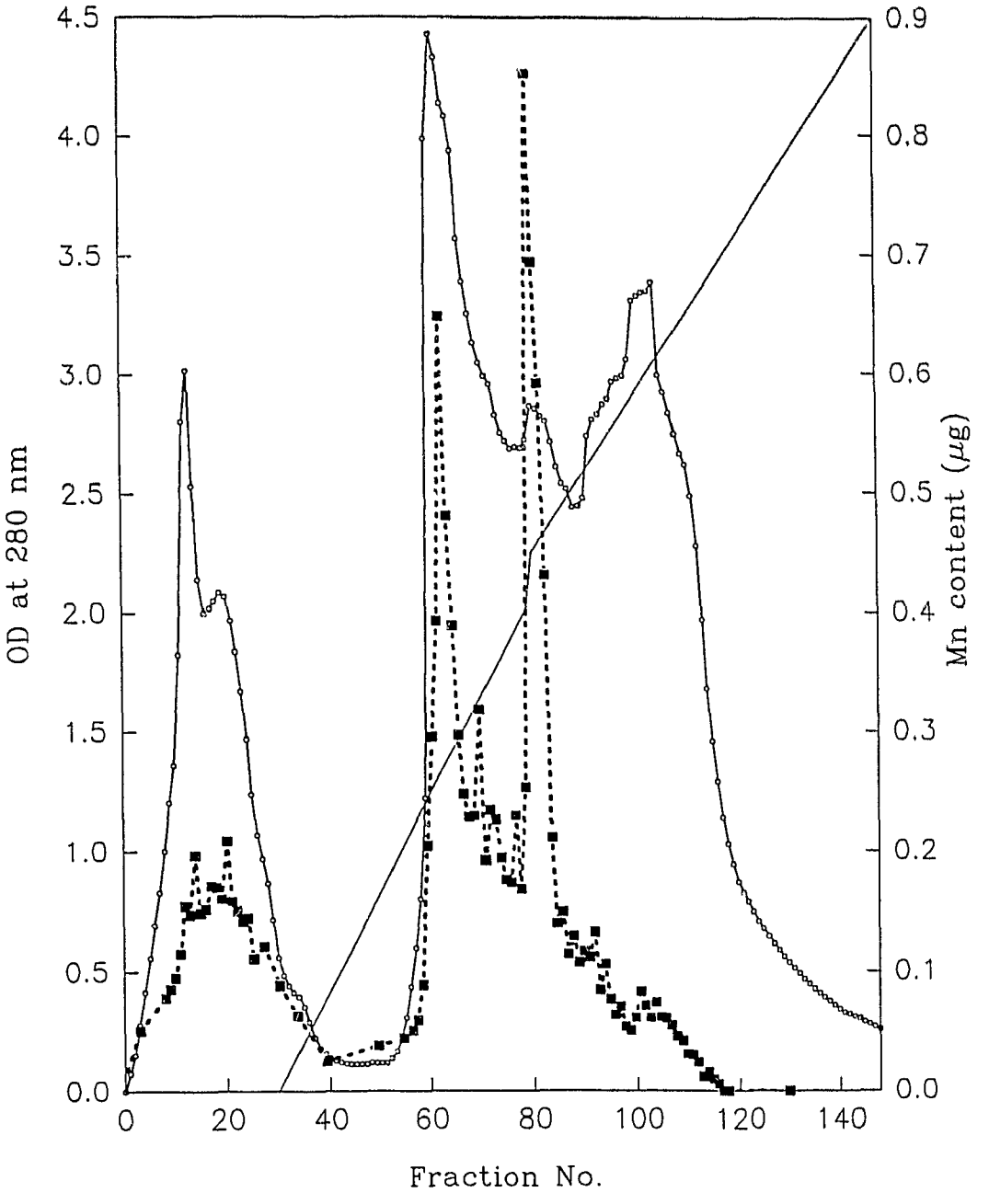


Fig. 5.7. Elution profile of proteins and manganese in cytosol cytosol on a DEAE-Sepharose column. Protein: (------), manganese: (■■■).

ammonium acetate, respectively. There was also an indication of two smaller Mn peaks at 0.4 M and 0.65 M salt concentrations. Peaks eluted at 0.3 and 0.5 M ammonium acetate had enrichment factors of Mn of 2 and 2.6, respectively, compared to the starting material. Anion exchange chromatography appears to be a very successful technique to study Mn-bound proteins in bovine kidney cytosol. The elution profile obtained is almost identical to the one reported by Jayawickreme [70]. He also detected three major Mn-containing protein peaks and one of them eluted before the salt gradient as it did in the present study. These observations may suggest that the microsomal fraction of bovine kidney does not contain any major Mn-binding proteins compared to the cytosol fraction. Only 5.9% of total Mn was detected in microsomes in this work and no information on studies on Mn-proteins from this fraction has been found in the literature. As mentioned earlier, majority of Mn investigations is carried out on the mitochondrial fraction of various tissues.

5.8 COPPER

A discussion of all publications and reviews on role of Cu in animal and man lies beyond the scope of this section. Several disorders and diseases in animals and humans have been recognized as being related to Cu deficiency. Two most severe ones are (i) Wilson's disease, characterized by a massive accumulation of copper mainly in liver and brain; and (ii) Menke's syndrome, a genetic disorder seen early in infancy and characterized by impaired intestinal

absorption of Cu and low levels of ceruloplasmin and Cu in serum.

In animals, Cu is concentrated in liver, brain, heart and kidney. Although muscle and bone contain intermediate levels, they account for 50-70% of total Cu in the body because of their overall greater mass [211]. Highly variable concentrations of Cu in various tissues suggest specific biochemical functions for this element in each organ. Subcellular distributions of Cu in rat liver at birth and in mature animals were studied by Gregoriadis and Soukes [228]. In the newborns, 80% of the total element was present in the mitochondrial and nuclear fractions; in adult rats, the supernatant fraction accounted for one half of total Cu followed by nuclei, mitochondria and microsomes. Also Jayawickreme [70] detected 48% of total tissue Cu in the cytosol fraction of bovine kidneys. Copper in rat brain and liver was investigated using ^{67}Cu radiotracer by Baerga *et al.* [229]. Similar subcellular distribution of Cu was found in both organs and the results agree well with those of Gregoriadis and Soukes. [228].

Copper present in body organs and fluids is never found in free ionic form. It is generally bound to proteins, peptides and amino acids. In blood, large amounts of Cu are present in erythrocytes and ceruloplasmin. Ceruloplasmin is a plasma glycoprotein, involved in Cu transport and in oxidation processes [230]. Copper in erythrocytes is mainly associated with superoxide dismutase, an enzyme responsible for destroying superoxide radical [211]. This is a Cu/Zn metalloenzyme of MW of 32 kDa consisting of two identical subunits each containing one atom of Zn and Cu.

One of the most studied cuproproteins is metallothionein (MT), a low-MW protein containing Zn and Cu and involved in mechanisms preventing heavy metal toxicity. This protein has the ability to bind a large number of metal ions because of high cysteine content. Studies have been reported on the synthesis of MT enhanced due to Cu intoxication [231], [232]. The distribution of Cu and MT in subcellular fractions of rat liver was studied by Sakurai *et al.* [233]. Both the protein and Cu were found to be concentrated mainly in cytosol followed by nuclei, mitochondria and microsomes.

Subcellular distribution of Cu in bovine kidneys and further studies of the supernatant fraction were done by Jayawickreme [70]. Different separation and purification techniques provided evidence for the existence of one Cu-binding protein in the kidney supernatant. Its MW (estimated using gel filtration) was 30 kDa, pI was 5.0 and the resemblance of this protein to the enzyme superoxide dismutase was suggested.

The concentration of Cu in the homogenate of bovine kidneys was 2.58 ± 0.49 ppm as shown in Table 5.10. Copper was most abundant in the nuclei fraction (46%) followed by mitochondria, cytosol, lysosomes and microsomes. The highest amount of Cu was found in the cytosol fraction by most researchers [229], [70] although the nuclei fraction was the richest in Cu in younger animals [228]. Only a small part of Cu present in cytosol was dialysable (7%, Table 5.1), which is in good agreements with data reported by other researchers [70], [228].

Table 5.10

Copper in homogenate and in subcellular fractions of bovine kidneys

Subcellular Fraction	Concentration (ppm)[a]	Distribution (%)
Homogenate	2.58 ± 0.49	(100)
Nuclei	1.19 ± 0.13	46.1 ± 5.1
Mitochondria	0.42 ± 0.14	16.3 ± 5.6
Lysosomes	0.19 ± 0.06	7.3 ± 2.5
Microsomes	0.10 ± 0.04	4.0 ± 1.4
Cytosol	0.40 ± 0.07	15.4 ± 2.7

[a] expressed as µg per g of wet sample.

The effect of ammonium sulfate precipitation on Cu-protein complexes in cytosol are presented in Tables 5.3 and 5.5. Before discussing any results, however, it is important to recall Table 5.2 where data on impurities detected in solid ammonium sulfate are presented. Concentration of Cu was very high in the solid chemical (0.76 ± 0.08 ppm) and a 4 M salt solution would contain approximately 0.4 ppm of this element. It is therefore highly possible that proteins might bind some of the Cu present externally in solution. This could be the reason why Cu-to-protein ratios are close to 1 in all precipitates (from 0.94 to 1.23), and why the ratio in the final supernatant is high (2.56) (Table 5.5). Also, total recovery of Cu was 107% which might be due to contamination. Ammonium

sulfate precipitation could be a very useful method as an initial step for the purification of Cu-containing proteins from crude cytosol provided there are no external Cu contaminating the sample. Copper in physiological fluids is present almost exclusively as attached to macromolecules and not as a free ion [211] which is due to the very high avidity of amino acids and proteins for Cu. For this reason, any free Cu present in solution will likely be complexed by protein leading to some erroneous results.

Fractionation of Cu-containing proteins on a DEAE-Sepharose column is shown in Fig 5.8. Copper eluted as a sharp, single peak at 0.3 M salt concentration giving 70 µg of this element in total and 82% recovery. Concentration of Cu in this peak increased by 4 compared to that in the initial sample. Ion exchange chromatography seems to be an excellent technique for the purification of Cu-containing proteins. Elution of this protein almost immediately after the application of salt gradient indicates its weak binding to the column and higher pI. Jayawickreme [70] also separated one major Cu-binding protein after anion exchange chromatography. That protein eluted at a very low salt concentration (0.1 M), but there is not sufficient data to compare these two proteins.

5.9 ZINC

Zinc is one of the most abundant trace elements in living organisms. Its essentiality for lower forms of life was discovered in the 19th century and for

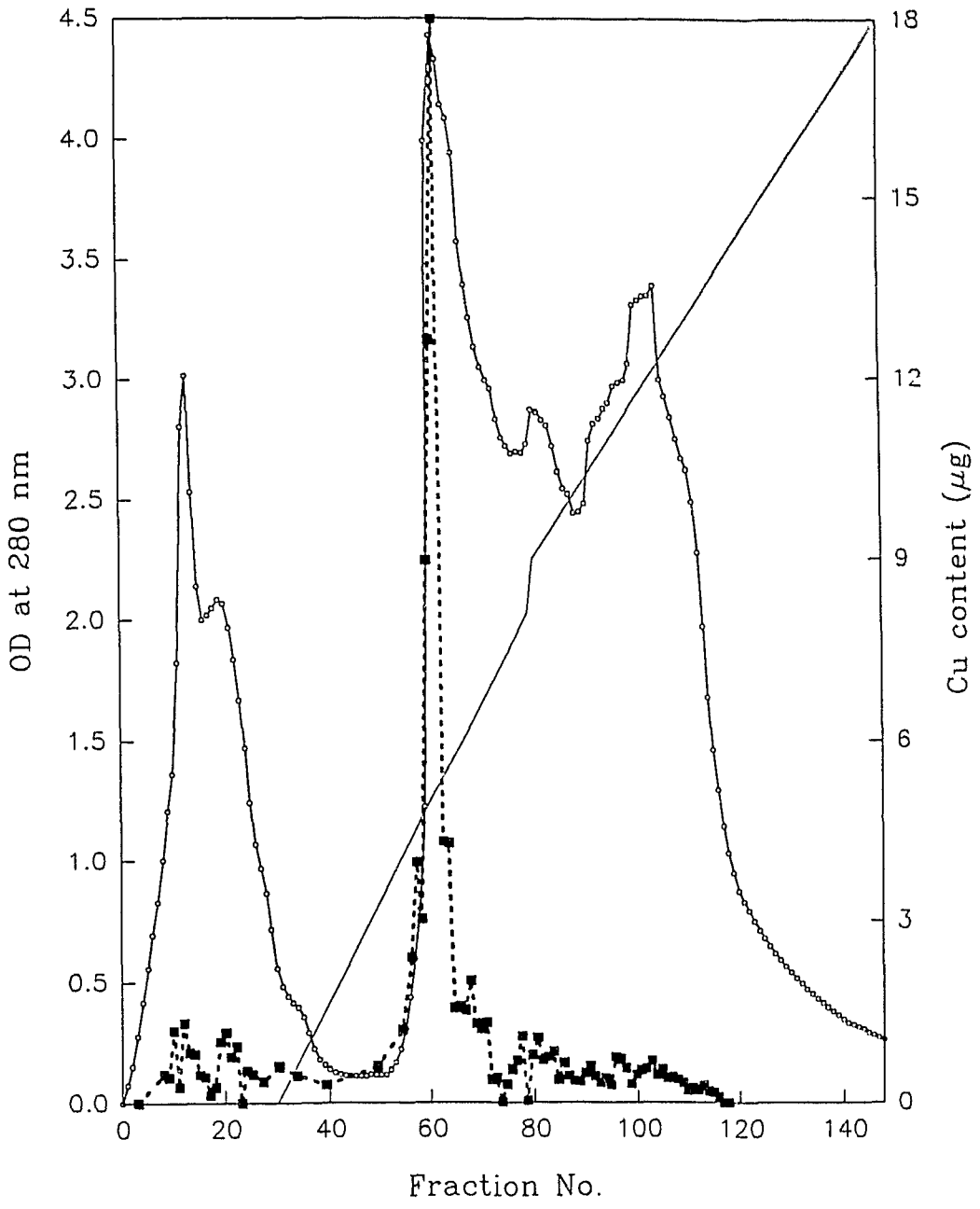


Fig. 5.8. Elution profile of proteins and copper in cytosol on a DEAE-Sepharose column. Protein: (------), copper: (■■■).

animals in 1930s. Acquired deficiency states in humans due to inadequate intake of Zn were first recognized in 1966 by Prasad *et al.* [42] as hypogonadal dwarfism in Egyptian and Iranian villagers. These conditions were reversed by oral Zn supplementation. It is now known that Zn is an essential component of more than 200 metalloenzymes isolated from different species. The element plays important roles in many processes, including synthesis of nucleic acid and protein, carbohydrate and energy metabolism, cell membrane stabilization, the immune system, gonadal function and others.

An average human body contains about 3 g of Zn. Retina, prostate, bone, muscle, kidney and liver are richest in Zn with concentrations ranging from 140 to 570 ppm. In terms of total body content, 62% of Zn is present in muscles, 28% in bone, 1.8% in liver and 0.1% in plasma [234]. Different organs accumulate Zn in different subcellular fractions, usually non-uniformly. Bovine pineal gland was studied by Awad *et al.* [235]. The highest concentration was found in the nuclei and cytosol (0.3 ppm) and then in mitochondria and microsomes. The subcellular distribution of Zn in monkey testis was also non-uniform with microsomes having the highest concentration [236]. In bovine kidneys [70], on the other hand, all fractions had approximately equal concentration (about 100 ppm) and 45% of total Zn was found in cytosol. In blood plasma, Zn concentration was determined as 1.12 ± 0.12 ppm [42] and in blood platelets as 46.3 ± 11.3 ppm [237]. Zinc levels in serum and urine are monitored to evaluate its status in the body, but many factors besides dietary Zn intake may control these values because the

bioavailability of Zn is influenced by the presence of other trace elements and components in a diet [222].

The first Zn-containing enzyme - carbonic anhydrase from erythrocytes - was discovered in 1940 [238]. Cu [illegible], Zn metalloenzymes are found in each class of the six categories of enzymes specified by the IUB (International Union of Biochemistry); it is the only metal for which this is the case. Most recently, Zn proteins have been demonstrated to be involved in transcription and translation of genetic material [239].

It has been already mentioned that metallothioneins (MT) predominantly bind Cd, Cu and Zn. Harford and Sarkar [240] studied the synthesis of MT in a type of renal cells following exposure to Cd and Zn. The induction of MT by each metal reached a maximum which could not be exceeded by increased level of the same metal. When the two metals were administrated simultaneously, however, an additive induction of MT was observed. The saturable nature of the induction of MT for each metal suggested that the mechanism of induction of MT is different for Cd and Zn. Induction of the hepatic metallothionein in rat after injection with $ZnSO_4$ was also studied [235].

Zn-binding proteins from bovine kidney supernatant (obtained by centrifugation at 30 000g for 25 min) were examined by Jayawickreme [92]. The presence of at least four Zn-proteins of MWs of >300 kDa, 260 kDa, 89 kDa, and 27 kDa were reported using gel filtration chromatography. Ion exchange chromatography and isoelectric focusing experiments also revealed four Zn-

containing fractions. Cadmium-binding proteins were also studied but the metals did not coelute from any of the two columns. On the basis of their MWs and pI values none of these proteins could be classified as metallothionein.

The concentrations of Zn in homogenate and subcellular fractions of bovine kidney are shown in Table 5.11. In homogenate, the concentration was 21.9 ± 3.4 ppm and nuclei and cytosol had the highest metal concentrations among all fractions: 8.29 ± 0.92 ppm and 7.77 ± 1.46 ppm, respectively. These two fractions were the most abundant in Zn (58.8% in nuclei and 35.5% in cytosol). Similar results were reported by Awad *et al.* [235], but, in general, the distribution of Zn in subcellular fractions varies from species to species and from organ to organ. An effect of dialysis on Zn in the cytosol fraction is shown in Table 5.1. Unlike the results reported by Jayawickreme [70] who detected 29.3% of Zn in the dialysate, only 6.3% of Zn was present in cytosol in a dialysable form.

Zinc-binding proteins were fractionated by ammonium sulfate precipitation. Zn was present in ammonium sulfate as a contaminant. As shown in Table 5.2, its concentration in this salt was 0.95 ppm. In a 4 M solution, it gives a concentration of approximately 0.5 ppm which is about 15 times lower than the concentration of Zn in cytosol (Table 5.1). Even if a large part of this Zn had complexed with proteins during the precipitation process, most likely no erroneous results would have been produced at this low Zn level. As presented in Table 5.3, majority of Zn was precipitated by 2- and 3-M salt solutions, the percentages being 42.8 and 25.3, respectively. An overall recovery of Zn was 87%. The calculation

of element-to-protein ratio (Table 5.5) showed that 1 M ammonium sulfate would precipitate Zn-containing proteins most effectively. These results indicated the presence of three different Zn-proteins that precipitated at various concentrations of ammonium sulfate.

Table 5.11

Zinc in homogenate and in subcellular fractions of bovine kidneys

Subcellular Fraction	Concentration (ppm)[a]	Distribution (%)
Homogenate	21.9 ± 3.4	(100)
Nuclei	8.26 ± 0.92	58.8 ± 6.1
Mitochondria	2.56 ± 0.66	34.5 ± 6.0
Lysosomes	1.71 ± 0.57	26.1 ± 11.2
Microsomes	1.91 ± 0.31	6.6 ± 2.5
Cytosol	7.77 ± 1.46	35.5 ± 6.7

[a] expressed as µg per g of wet sample.

The elution profiles of Zn and proteins after ion exchange chromatography on a DEAE-Sepharose column are presented in Fig. 5.9. A small amount of Zn eluted along with the material that did not bind to the column and then it separated into three peaks eluted by 0.3, 0.5 and 0.65 M ammonium acetate, respectively. The first peak was most abundant and Zn levels in these fractions increased by

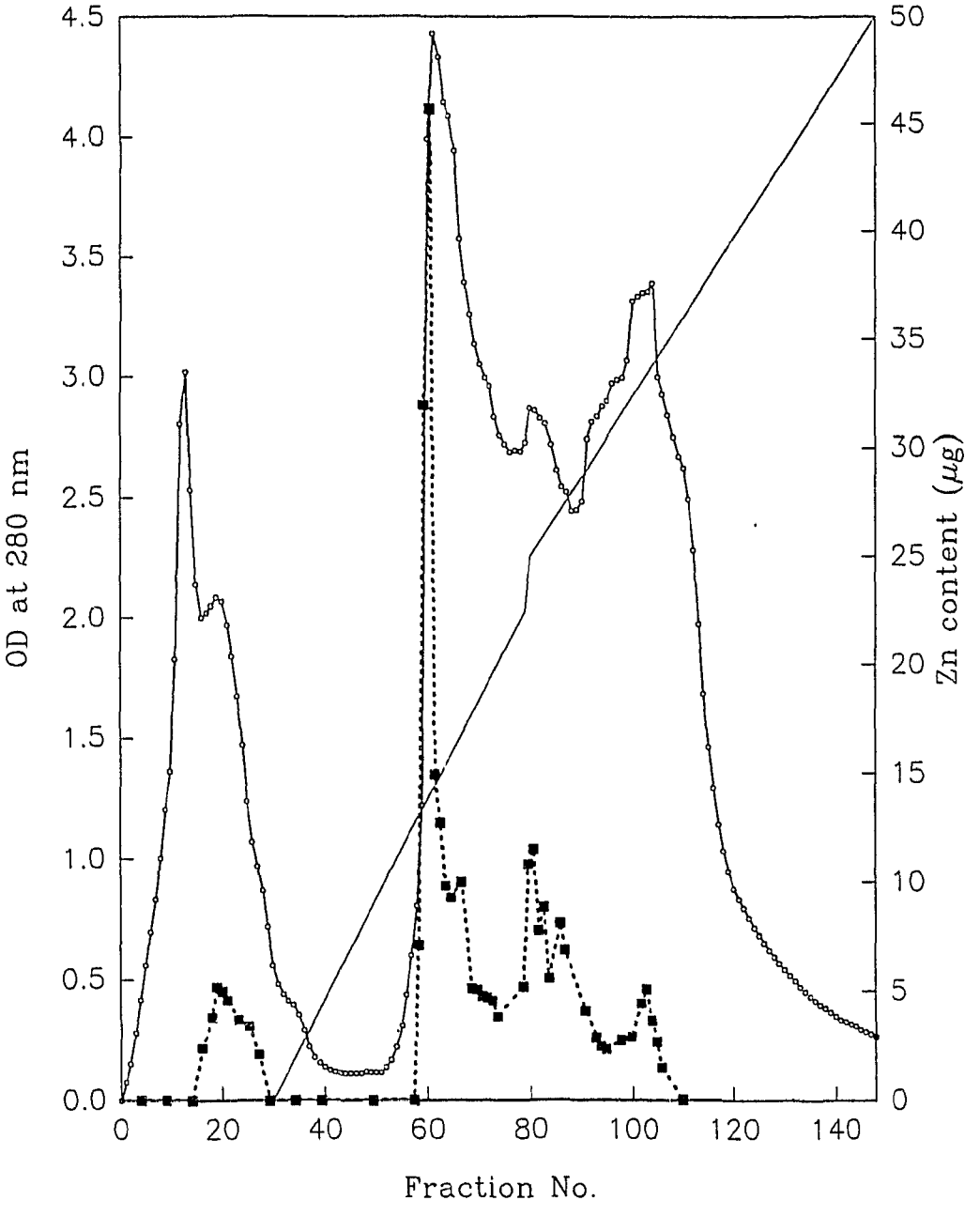


Fig. 5.9. Elution profile of proteins and zinc in cytosol on a DEAE-Sepharose column. Protein: (------), zinc: (-■-■-■-).

a factor of 2 over that in the starting material. Jayawickreme [92] reported the presence of four major Zn-proteins and possible existence of four minor peaks in kidney supernatant. It is possible that some of the Zn-proteins detected by him come from the microsomes that are present in the supernatant fraction. In his work, the main Zn peak eluted from a DEAE-Sepharose column without binding. Here, the majority of Zn eluted right after applying the salt gradient. It is unlikely that the highest Zn peak is a metallothionein. Its early elution form the column indicates higher pI value, and the majority of purified metallothioneins have pI values < 5 [241], [242]. On the other hand, one or both of the other two Zn-containing proteins could be metallothionein(s) as the late elution from the column indicated lower pI values of these proteins. Additional investigations need to be done in order to confirm these postulates.

5.10 SUMMARY

A combination of bioanalytical and neutron activation methods which were developed to study Se-binding proteins in bovine kidneys was also applied to investigate the association of several other protein-bound trace elements in this organ. The elements included As, Br, Ca, Cl, Cu, F, Fe, I, K, Mg, Mn, Mo, Na, Rb, S, V and Zn. Differential centrifugation was utilized to separate the homogenate into five cell fractions as described in Chapter 3. The cytosol fraction was studied in more detail. Dialysis was used to determine the extent of binding of these

elements to macromolecules. Stepwise ammonium sulfate precipitation fractionated protein-bound trace elements and could be effectively used as an initial step in the purification scheme. Contamination coming from salt created problems in selected cases and the use of an ultrapure chemical would be of great advantage. Ammonium acetate was selected to replace NaCl as an eluting agent. Protein-bound As, Cu, I, Mn, V and Zn were investigated using ion exchange chromatography.

6. CONCLUSIONS AND RECOMMENDATIONS

The main objective of this work was to investigate selenoproteins present in bovine kidney cytosol fraction. Additionally, selected protein-bound trace elements in cytosol and other fractions of the kidney were studied. Several biochemical methods for protein purification and characterization and an instrumental neutron activation analysis (INAA) method for elemental determination were developed and successfully applied.

Twenty eight elements were detected in various biological materials using INAA. A combination of four different irradiation (t_i), decay (t_d) and counting (t_c) times were selected for the multielement analysis of the samples.

Both internal and external quality assessment steps were followed to ensure the quality of the results obtained. These steps included: (i) selection of appropriate target isotopes and nuclides; (ii) correction for spectral interferences and competing reaction interferences; (iii) measurements of analytical blank; (iv) optimization of sample geometry; (v) analyses of replicate samples under identical conditions; (vi) construction of control charts for comparator standards; and (vii) analyses of standard reference materials. Precision of the INAA method was found to be very good and well within the warning limits (± 2 SD) on the quality control charts. Accuracy was also satisfactory as 82% of all elements determined agreed within ± 10% with their certified values.

Determinations of concentrations of such elements as Al, Mn, F, and Hg suffer from spectral interferences and interferences occurring during activation (competing reactions). These interferences were recognized and necessary correction methods were applied except for the Mn determination where the contribution from the interfering Al reaction was found to be less than 3%.

Detection limits of the elements observed in bovine kidney and its homogenate were calculated according to Currie's method [65]. The values obtained for kidney were quite high due to high Na and Cl content of the samples. A single washing of disrupted kidney tissue during the homogenate preparation removed majority of Na and Cl and allowed for a significant decrease in the detection limits of the elements investigated.

In summary, the INAA method developed was found to be suitable for the multielement analysis of various biological samples and could be applied to other similar studies.

Bovine kidney was fractionated to five subcellular fractions by differential centrifugation. Certain enzymes are known to be located predominantly in one part of the cell (*i.e.* fraction) and the possibility of cross-contaminations among fractions could be evaluated by assaying activities of such marker enzymes. In this work, the following enzymes were assayed: (i) glutamate dehydrogenase (mitochondrial marker); (ii) acid phosphatase (lysosomal marker); (iii) glucose-6-phosphatase (microsomal marker); and (iv) lactate dehydrogenase (cytosolic marker). The results indicated that the mitochondrial, lysosomal and microsomal

fractions could be cross-contaminated. In future studies, the separation procedure would need to be improved in order to achieve a higher degree of purity. On the other hand, cytosol turned out to be fairly free from activities of other enzymes and rich in the activity of its marker enzyme, lactate dehydrogenase. Studies on protein-bound trace elements in cytosol were conducted without employing any additional purification steps.

The diverse metabolic functions of Se in living organisms has been recognized relatively recently. A few selenoproteins discovered so far cannot account for the total Se content in the body and for the evidences of its essentiality and toxicity. Selenium levels not associated with glutathione peroxidase activity in the kidneys is one of the highest compared to that in other body fluids and tissues. In this work, the cytosol fraction was found to contain up to 10% of total Se determined in the kidney. Only 8% of it was dialysable implying the existence of protein-bound Se. Three Se-containing proteins, called Selenoprotein A1, P1 and P2, were purified and characterized in this fraction.

Selenoprotein A1 was purified using chloroform-ethanol precipitation, DEAE-Sepharose chromatography and gel filtration chromatography. Its MW and pI were 71 kDa and 5.5, respectively. Selenoproteins P1 and P2 were purified using ammonium sulfate precipitation, DEAE-Sepharose chromatography and gel filtration chromatography. Their MWs and pI values were: for Selenoprotein P1 - MW of 62 kDa and pI of 5.4; and for Selenoprotein P2 - MW of 48 kDa and pI of 4.9. The SDS-PAGE experiments showed that all three selenoproteins consisted

of one subunit. These proteins have not yet been reported in the literature. Future studies involving the determination of Se as amino acid selenocysteine and search for any enzymatic activities of these proteins may reveal unique biological functions of Se in kidneys. It would be also interesting to investigate other subcellular fractions for the presence of these 3 selenoproteins.

INAA allows for multielement analysis of the samples investigated and this feature was readily utilized to study various minor and trace elements in bovine kidney subcellular fractions in more detail. Aluminum, Br, Cl, Cu, F, Fe, K, Mg and Zn were found predominantly in the nuclear and cytosolic fractions. Chromium, Mn and V were abundant in the mitochondrial fraction. Selenium was detected mostly in the nuclear fraction (80%). Microsomes contained higher levels of only two elements: Fe (15%) and Zn (8%). For almost all elements, the highest element-to-protein ratios were found in the cytosol. Elements from this fraction were examined further using dialysis, ammonium sulfate precipitation and ion exchange chromatography.

Dialysis of cytosol showed that only about 10% of Cu, Fe, Mo, S, Se and Zn were dialysable. These elements must therefore exist in cytosol as attached to the macromolecules. About 35% of Ca and 24% of Mg were dialysable. More than 90% of Br, Cl, F, K, Na and Rb dialysed; they could be present in cytosol as free ions or small molecules.

Ammonium sulfate precipitation of cytosol gave satisfactory results for the majority of the elements investigated. The overall recovery of protein-bound trace

elements ranged between 82 and 100%. Elements were found to be non-uniformly distributed in protein fractions precipitated by various concentrations of the salt. The experiment could therefore be successfully applied as an initial step in the fractionation of protein-bound trace elements. Since large quantities of salt must be used for the precipitation of proteins, a salt free of elemental contaminants should be applied. In this work, results obtained for Cr Cu and Zn could have been affected by the presence of these elements in salt as contaminants.

Protein-bound As, Cu, I, Mn, V and Zn were investigated further by ion exchange chromatography. Ammonium acetate has been selected as an eluting agent for this experiment. Arsenic separated into two peaks eluted from the column by 0.25 and 0.3 M salt. Iodine was detected in at least 6 peaks suggesting the presence of several I-containing proteins in kidney cytosol. Vanadium was present in three protein peaks with an overall recovery of 77%. Protein-bound Mn separated on a column into two peaks eluted by 0.3 and 0.5 M salts, respectively. Copper eluted from the column as a single peak at 0.3 M ammonium acetate which gave 82% recovery of the element and 4-fold increase in the concentration over that in the initial sample. Ion exchange chromatography appeared to be a very effective method for the purification of Cu-containing proteins. Zinc eluted from the column as bound to at least three proteins eluted at 0.3, 0.5 and 0.65 M salt. The first peak accounted for the majority of the element recovered from the column. Further purification and characterization of these protein-bound trace elements may be important in understanding their

biological functions in kidneys. Especially studies on V and As would be of great importance as very little is known so far on the association of these elements with proteins and their functions in living organisms.

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