2. Materials and Methods

In the studies element analytical methods and radiotracer techniques have been combined with biochemical separation procedures. All methods are described below; more details are shown in the experimental part of this work.

2.1 Materials

2.1.1 Chemicals

In principle only chemicals described as "pro analysis" or better were used in all experiments. A Milli-Q system (Millipore, Eschborn, Germany) was used to prepare ultra-pure deionised water (conductance $\geq 10\ M\Omega \cdot cm^{-1}$).

2.1.2 Radiotracer ^{75}Se

Animals and cultured cells were labeled *in vivo* by administration of [^{75}Se]-selenite. This radioactive isotope has a half-life of 120.6 days and decays to non-radioactive ^{75}As by electron capture. The excited ^{75}As emits γ-rays which can be measured by means of a scintillation detector or by autoradiography.

The radionuclide ^{75}Se was produced in the nuclear reactor BER II of Hahn-Meitner-Institute (Berlin, Germany) or in the nuclear reactor of the University of Missouri (Columbia, USA), after irradiation of enriched ^{74}Se (elemental form) with neutrons for several months. In this way a radionuclide with a high specific activity was produced. The elemental selenium was dissolved in nitric acid and in this way oxidized to selenite. For the injection the [^{75}Se]-selenite solution was neutralized with sodium hydroxide and diluted in saline solution.

2.1.3 Radiotracer ^{73}As

The radioisotope ^{73}As with a half-life of 80.3 days was used for the labeling of animals and culture cells. This radionuclide decays by electron capture to non - radioactive ^{73}Ge that is stabilized by γ-ray emission. This radioisotope was produced by a spallation process after bombarding of rubidium chloride with high energy protons in the Oak Ridge National Laboratory (Tennessee, USA). The element was dissolved in 0.1 M hydrochloric acid. For injection the [^{73}As]As^{V} solution was neutralized with sodium hydroxide and than diluted the saline solution.

2.1.4 Experimental animals

Wistar rats (Charles River, Sulzfeld, Germany) were used for the experiments. The animals were kept in an artificial day-night rhythm of 12 hours in cages made of polycarbonate and were nourished with special diets and deionised water ad libitum. The rats were fed either a low selenium diet (about 10 µg Se/kg) for several generations or a selenium-adequate diet which was produced from the basal diet by adding 300 µg Se/kg as sodium selenite. The basal diet consisted of several substances listed in the Table 2-1. The concentrations of trace elements present in both types of diet were measured by means of INAA and the results are shown in the Table 2-2.

Table 2-1 Concentration of substances present in the basal diet (ICN Pharmaceuticals, Cleveland, USA).

Substance	Concentration [g/kg]	Substance	Concentration [g/kg]
Sucrose	590	Choline chloride	75
Torula yeast	300	Vitamin D	0.125
Tocopherol poor fat	50	Glucose	900.5
Minerals composition	50	Folic acid	0.09
D,L-methionine	0.3	Inositol	5
D,L-α tocopherol acetate	0.2	Menadion	2.25
p- aminobenzoic acid	5	Niacin	4.25
Vitamin B_{12}	0.00135	Riboflavin	1
Biotin	0.02	Pyridoxine-HCl	1
Calcium panthothenate	3	Thiamine-HCl	1

Table 2-2 Concentrations of selenium, arsenic, zinc, cobalt and iron in the diets analyzed by means of INAA.

Elements	Selenium-deficient diet [mg kg^{-1} dry weight]	Selenium-sufficient diet [mg kg^{-1} dry weight]
Se	< 0,03	0,31 ± 0,04
As	< 0,26	< 0,26
Zn	67 ± 0,5	64 ± 0,5
Co	0,096 ± 0,002	0,097 ± 0,002
Fe	357 ± 4	337 ± 4

For the tracer experiments the animals were labeled in vivo for 3 to 7 days with ^{75}Se by i.p. administration of 0.5 ml of the saline solution with a ^{75}Se activity of 18.5 MBq and with ^{73}As for 2 days by i.p. injection or oral administration of 0.6 ml of the saline solution with 18 MBq of ^{73}As.

2.1.5 Cell lines

A human lung carcinoma A-549 cell line (DSMZ GmbH, Germany), a normal human trachea cell line Hs 680.Tr (ATCC, USA) and a normal rat lung cell line L2 (ATCC, USA) were cultured for the experiments.

A-549 cells were established from an explanted lung tumor which was removed from a 58-year old Caucasian man in 1972. The cells are epithelial cells, growing adherently as monolayer in 90% Dulbecco's MEM Medium with 10% FBS. The cultures were splat confluent 1:5 to 1:10 every 4-7 days using Trypsin-EDTA. The cells were incubated at 37°C in 5% $C0_2$ in a humidified air atmosphere until they were ready to be subcultured.

Hs 680.Tr cells were established from a 16-year old Caucasian female. These cells are tracheal fibroblasts. They were cultured in 90% Dulbecco's Modified Eagle's Medium with 10% FBS and splat 1:2 to 1:4 every 4-6 days using a Trypsin-EDTA solution. The cells were incubated at 37°C in 5% $C0_2$ in a humidified air atmosphere.

L2 cells were established from rat lung (*Rattus norvegicus*). They are morphologically classicized as epithelial cells. The cells were growing adherently as monolayer in 90% Ham's

F12K Medium with 10% FBS and splat 1:2 every 2-3 days. The cells were incubated at 37°C in 8% $C0_2$ in a humidified air atmosphere.

HepG2 cells were established from a 15-year old Caucasian male. The cell line was derived from the hepatocellular carcinoma cell line, HepG2. The parental cells were stably transfected at passage 48 with human cholesterol 7 alpha-hydroxylase (CYP7) minigene/Luciferase construct. HepG2 are morphologically classicized as epithelial cells. They were grown adherently as monolayer in 90% Dulbecco's MEM Medium with 10% FBS. The cultures were splat confluent 1:5 to 1:10 every 4-7 days using Trypsin-EDTA. The cells were incubated at 37°C in 5% $C0_2$ in a humidified air atmosphere until they were ready to be subcultured.

2.1.6 Antibodies

For the immunoassay the following antibodies were used: Anti-Glutathione Peroxidase 1 (Gpx1) polyclonal antibody from Acris Antibody GmbH (Hiddenhausen, Germany); Anti-SelT (18 kDa selenoprotein) peptide antibody produced by Biogenes (Berlin, Germany); Anti-Selenoprotein P, a peptide antibody kindly provided by Dr. Ulrich Schweizer (Charite, Berlin); Anti-Thioredoxin reductase 1 (Trx1) kindly provided by Prof. Dr. Katja Becker (Justus-Liebig University of Giessen).

2.2 Methods

2.2.1 Element analytical methods for the determination of trace elements

2.2.1.1 Neutron activation analysis

Instrumental neutron activation analysis (INAA) was used for the quantitative multi-element analysis of trace elements present in the lung and trachea and their subcellular fractions. The samples were lyophilized at -20°C, homogenized, weighed in a plastic container or quartz ampoule and sealed in them. For the analysis of the trace elements in the subcellular fractions the samples were prepared as described in 2.2.5.2. The ampoules were placed either in the core or near the core of the nuclear reactor BER II of the Hahn-Meitner-Institut and irradiated for a suitable period of time. After the irradiation the samples were allowed to decay and then gamma-ray spectra were measured using a high purity germanium detector. The energies of

the delayed gamma rays were used for qualitative analysis of the sample, and the intensity of the gamma rays of a specific energy was used to determine the amount of an element in the sample [107]. Final results were obtained by correcting for decay, sample size, counting time and irradiation time. The relation between the radioactivity of the irradiated isotope and its amount in the samples can be described by following equation:

$$A = \varphi \, \sigma \, I_\gamma \, n \, N_A \, (1\text{-}e^{-0{,}693 \, t_B \, / \, t_{1/2}})$$

A = absolute activity of isotope ^{A+1}Z in sample (Bq)
Φ = neutron flux density (neutrons·cm^{-2}·sec^{-1})
σ = neutron capture cross section (cm^2) for isotope ^{A}Z
n = amount of isotope ^{A}Z in sample (mol)
I_γ = relative isotopic abundance
N_A = Avogadro number
t_B = irradiation time (s)
$t_{1/2}$ = decay time (s) of the isotope

Some characteristics of the radionuclides used in INAA are listed in Table 2-3. The comparator standard approach was employed with this method. Standards were irradiated and counted along with the samples. These standards contained known amounts of the elements to be determined. The quantitative analysis was carried out by evaluation of the standard reference material bovine liver (NIST, Gaithersburg, USA) and a multi element standard (Merck, Darmstadt, Germany).

With some samples, were the samples mass was very low, it was not possible to split them for different irradiations, optimized for the half-lifes of the radionuclides used for analysis. However, INAA after repeated irradiation allowed the determination of several elements with different half-lifes times, such as arsenic, rubidium, manganese, iron, zinc and selenium in the same samples. The blank values of the elements in Tris buffer used in the sample preparation were likewise measured. They were found to be below the detection limit.

Table 2-3 Some characteristics of the radionuclides used in the analysis of the elemental concentrations

Element	Nuclide	Half-life	E_γ [keV]
Mn	Mn-56	2.6 h	846.8; 1810.8
As	As-76	26.3 h	559.1
Rb	Rb-86	18.7 d	1076.7
Fe	Fe-59	44.5 d	1099.3; 1291.6
Se	Se-75	120.6 d	136.0; 264.7
Zn	Zn-65	244.1 d	1115.5
Cr	Cr-51	27.7 d	320.1
Co	Co-60	5.3 y	1173.2, 1332.5
Na	Na-24	14.9 h	1368.6
K	K-42	12.4 h	1524.6

Manganese was analyzed via ^{56}Mn after irradiation of the samples at a neutron flux density of $6{,}6 \cdot 10^{12}$ $cm^{-2}s^{-1}$ for 30 minutes. After a cooling time of approx. 1 hour the gamma ray spectra were measured for 1 hour. The samples were irradiated again at the same reactor position for the determination of arsenic, potassium and sodium via the medium-lived ^{76}As, ^{42}K and ^{24}Na. After a cooling time of approx. 1 hour the samples were measured for 1 hour. After a decay period of 3 weeks the third irradiation was performed at a neutron flux density of $1{,}6 \cdot 10^{14}$ $cm^{-2}s^{-1}$ for 24 hours for the determination of selenium, iron, zinc, rubidium, chromium and cobalt via the long-lived radionuclides ^{75}Se, ^{59}Fe, ^{65}Zn, ^{89}Rb, ^{51}Cr and ^{60}Co. Following a cooling time of 1 month the gamma ray spectra were measured for 4 hours.

The reasons for choosing neutron activation analysis (NAA) were its non-destructive character and sufficiently low detection limits even with small sample masses. For other powerful methods such as atom absorption spectrometry (AAS) or inductively coupled plasma mass spectrometry (ICP-MS) the samples had to be dissolved or digested, always be in risk of contamination.

2.2.1.2 ICP-MS and HPLC

Inductively coupled plasma mass spectrometry (ICP-MS) was used for the determination of several trace elements in the cytosolic proteins of the lung and trachea after separation by size exclusion chromatography (SEC). The quadrupole-ICP-MS HP 4500 and HP 7500C from

Hewlett Packard/Agilent Technologies (Waldbronn, Germany) were used. For the measurement of trace elements in the cytosolic fraction the ICP-MS system was coupled to the HP 1100-HPLC column. The instrumental set up of the ICP-MS system is described in the Table 2-4.

Plasma is defined as a gas consisting of ions, electrons, and neutral particles. In ICP-MS, the plasma is formed from argon. The plasma is used to atomize and ionize the elements in a sample. The resulting ions then pass through a series of apertures (cones) into the high vacuum analyzer. The isotopes of the elements are identified by their mass-to-charge ratio (*m/e*). The intensity of a specific peak in the mass spectrum is proportional to the amount of that isotope (element) in the original sample. The inductively-coupled plasma is a very aggressive ion source. Because the source operates at temperatures of 7000 K, virtually all molecules in a sample are broken up into their component atoms. In ICP, a radio frequency (RF) signal is fed into a tightly wound, water-cooled, coil where it generates an intense magnetic field. In the center of this coil is a specially made glass or quartz plasma torch where the plasma is formed. The plasma is generated in the argon by "seeding" the gas with a spark from a Tesla unit. When the spark passes through the argon some of the argon atoms are ionized and the resulting cations and electrons are accelerated toward the magnetic field of the RF coil. Through a series of inelastic collisions between the charged (cations and electrons) particles and neutral argon atoms, a stable high temperature plasma is generated. The concentrations of electrons, cations and neutral species in the plasma very quickly reach equilibrium, after which the plasma will remain 'lit' as long as the RF field is maintained and there is a constant supply of argon into the plasma. The plasma torch is designed in such a way as to allow a sample to be directly injected into the heart of the plasma. The sample consists of a fine aerosol, which can come from type of sources including, but not limited to, nebulized liquids or ablated solids. As the sample aerosol passes through the plasma, it collides with free electrons, argon cations and neutral argon atoms. In this way any molecules initially present in the aerosol are quickly and completely broken down to charged atoms. These are usually in the M^+ state although a few M^{++} are also formed. Some of these charged atoms will recombine with other species in the plasma to create both stable and meta-stable molecular species (*e.g.* MAr^+, M_2^+, MO^+, etc.). Many of these molecular species are positively charged and are also transported into the mass analyzer along with the charged atoms (M^+ and M^{++}).

Table 2-4 Instrumental set up of the ICP MS system

Nebulizer	Babington Scott spray chamber
Plasma gas flow	Argon 15,2 l min^{-1}
Auxiliary gas flow	Argon 1 l min^{-1}
Nebulizer gas flow	Argon 0.9-1.1 l min^{-1}
Blend gas flow	Argon 0.1-0.25 l min^{-1}
RF power	1200 W
Acquisition time/ replicate	8,5505 s (36 isotopes)
Isotopes detected	^{24}Mg, ^{27}Al, ^{31}P, ^{34}S, ^{35}Cl, ^{42}Ca, ^{53}Cr, ^{54}Fe, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{64}Zn, ^{65}Cu, ^{67}Zn, ^{72}Ge, ^{72}Ge, ^{75}As, ^{77}Se, ^{82}Se, ^{85}Rb, ^{98}Mo, ^{103}Rh, ^{107}Ag, ^{109}Ag, ^{111}Cd, ^{114}Cd, ^{127}I, ^{133}Cs, ^{193}Ir, ^{200}Hg, ^{208}Pb
Integration time	0.25 s per point (pulse mode) 0.1 s per point (analog mode: ^{31}P, ^{35}Cl)

2.2.1.3 Graphite furnace atomic absorption spectrometry

Graphite furnace atomic absorption spectrometry (GFAAS) was applied for quantitative analysis of copper and nickel in the cytosolic and mitochondrial fractions of the lung and trachea using a 5100PC system from Perkin Elmer with Zeeman background correction and the graphite furnace HGA-600 with pyrolytically coated graphite tubes with a L'vov platform (PerkinElmer, Überlingen, Germany). These fractions were prepared as described (see 2.2.4.2). The mitochondria were sonificated for an efficient decomposition.

In AAS light of a specific wavelength is passed through the atomic vapor of an element of interest, and the attenuation of the intensity of the light as a result of absorption is measured. Quantitative analysis by AAS depends on: accurate measurement of the intensity of the light and the assumption that the radiation absorbed is proportional to the atomic concentration.
GFAAS instruments have the following basic features: 1. a source of light (lamp) that emits resonance line radiation; 2. an atomization chamber - graphite tube in which the sample is vaporized; 3. a monochromator for selecting only one of the characteristic wavelengths (visible or ultraviolet) of the element of interest; 4. a detector, generally a photomultiplier tube (light detectors that are useful in low-intensity applications), that measures the amount of absorption; 5. a signal processor-computer system (strip chart recorder, digital display, meter,

or printer). Figure 2-1 shows the schematic diagram of the basic components of a GFAA system.

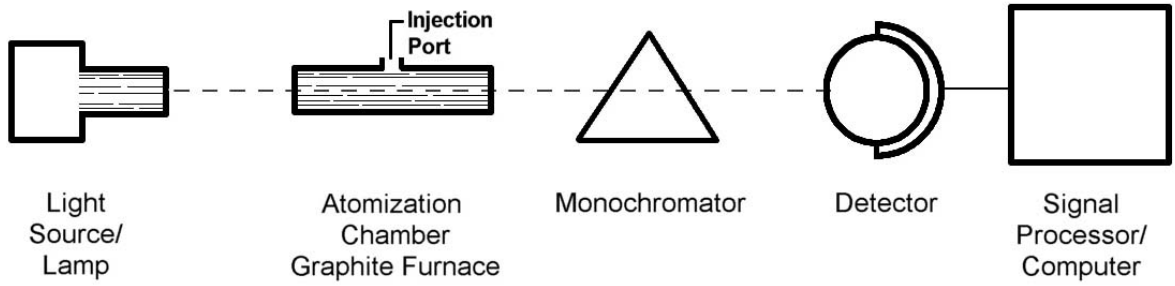

Figure 2-1 Diagram of the basic components of a graphite furnace atomic absorption spectrometer

The graphite furnace is an electro thermal atomizer system that can produce temperatures as high as 3,000°C with a graphite tube that is pyrolytically coated to minimize pitting and deterioration. The heated graphite furnace provides the thermal energy to break chemical bonds within the sample and produce free ground-state atoms present in the gas phase. Ground-state atoms absorb energy in the form of light and are elevated to an excited state. The amount of light energy absorbed increases as the concentration of the selected element in the gas phase increases.

The parameters of the measurement are shown in the Table 2-5. After the instrument had warmed up, a small aliquot, typically 10 - 20 µL, was placed, either manually or by means of an automated sampler, into the opening in the graphite tube. For the copper measurement 5 µl of the samples and 5 µl 0.1% triton was usually taken. In the case of nickel it was necessary to use Pd as $Pd(NO_3)_2$ as matrix modifier. Here the mixture consisted of 5 µl of the sample, 5 µl 0.1% triton and 10 µl $Pd(NO_3)_2$ (5 µg Pd). The blank values of the elements investigated in the Tris buffer were below detections limit.

Table 2-5 The measuring parameter of the GF AAS

	Copper	Nickel
Source of radiation	HCL[a] (15 mA)	HCL[a] (25 mA)
Wave length	324.8	232.0
Matrix modifier	-	5 µg Pd as $Pd(NO_3)_2$
Pre-atomization temperature	1000 °C	1300 °C
Atomization temperature	2300 °C	2500 °C
Calibration	Standard addition	Standard addition

[a]HCL: hollow cathode lamp

2.2.2 Determination of ^{75}Se and ^{73}As by means of a scintillation detector

For the determination of ^{75}Se and ^{73}As in the labeled tissues and cells a scintillation detector was used. A popular method for the detection of gamma rays involves the use of crystal scintillators. The general description of a scintillator is a material that emits low-energy (usually in the visible range) photons when struck by a high-energy charged particle. When used as a gamma-ray detector, the scintillator does not directly detect the gamma-rays, but the gamma-rays produce charged particles in the scintillator crystal which interact with the crystal and emit photons. These lower energy photons are subsequently collected by a photomultiplier tube (PMT). When gamma-rays pass through matter, they can undergo three basic processes: Compton scattering, photo absorption, or pair production. These processes create high-energy electrons or anti-electrons (positrons) which interact in the scintillator as charged particles. By adding up the energy collected in the surrounding photomultiplier tube, we can determine the energy of the gamma-ray in question. The scintillator is made of an alkali halide salt such as sodium iodide (NaI), which contains a small amount of thallium as an "activator".

2.2.3 Autoradiographic detection of the tracer in the gel after electrophoresis

An "Imaging Plate", a film like radiation image sensor, has been used for the determination of the labeled proteins in the gel after electrophoresis. The plate is a flexible image sensor with bunches of very small crystals of photo-stimulable barium fluorobromide containing a trace amount of bivalent europium ($BaFBr:Eu^{2+}$) as a luminescence center, uniformly coated on a polyester support film [108]. These phosphors can trap and store the radiation energy. The stored energy is stable until scanned with a laser beam (here the Helium-Neon-Laser in Phospho-Imager, Bas 1000, Fuji), which then releases the energy as luminescence [109].

2.2.4 Statistical analysis

All analytical results are expressed as means ± SD. The determined data were statistically analyzed using the Student's *t* – test to assess statistical significances. *P* – values < 0.05 were taken as significant.

2.2.5 Methods of protein chemistry

2.2.5.1 Protein assay of Bradford

The colorimetric protein assay, based on the Bradford method, was used for the measurement of the protein concentration in the samples. This assay is based on a shift in the absorbance maximum when Coomassie® Brilliant Blue G-250 dye reacts with protein. The Beer`s Law is applied for quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration. At the assay pH, the dye molecules are doubly protonated and are present as the red cationic dye form. Binding of the dye to protein stabilizes the blue anionic dye form, detected at 595 nm. Dye binding requires a protein containing an active basic or aromatic residues. The calibration curve was run each time a protein assay was performed. For this bovine serum albumin standard solutions were taken. Using the standard curve, the concentration of each sample was determined from its absorbance by interpolation [110].

2.2.5.2 Gel electrophoresis techniques

SDS-PAGE method of Laemmli [111]

For the analysis of proteins the SDS-PAGE method of Laemmli was applied. The electrophoresis was carried out using the vertical apparatus from Pharmacia (Pharmacia, Germany). The polyacrylamide gel consisted of the stacking gel and the separating gel. Cassettes were formed (120×160 mm) by two glass plates separated by spacers of the required thickness (1.5 mm) placed at the sides. The composition of the stacking gel and the separating gel are listed in the table 2-6.

Table 2-6 The composition of the solutions used in SDS PAGE

	Stacking gel	Separating gel
Acrylamide	5% (w/v)	15% (w/v)
N, N`methylenbisacrylamide	0.13% (w/v)	0.4% (w/v)
SDS	0.1% (w/v)	0.1%
Tris - HCl	125 mM pH 6.8	375 mM pH 8.8
TEMED	0.03% (v/v)	
APS	0.025% (w/v)	

Protein samples for SDS-PAGE were prepared by mixing them with 4% SDS, 20 mM DTT, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 12.5 mM Tris – HCl pH 6.8 in loading buffer and denaturizing for a few minutes at 95°C. The electrophoretic buffer was 50 mM Tris

– HCl pH 8.3, 200 mM glycine and 0.01% (w/v) SDS. The separation was carried out either for 12 h at a current of 12 mA or for 3h at 50 mA per gel. Protein molecular weight standards were purchased from Bio-Rad (Bio-Rad, Germany).

Tricine-SDS-PAGE method of Schägger and von Jagow [112]

For the analysis of the proteins with molecular masses below 30 kDa the tricine-SDS-PAGE of Schägger and von Jagow was applied. The only difference between this technique and that of Laemmli is the use of a spacer gel between the stacking gel and the separating gel and tricine instead of glycine. This allowed a better resolution of the small proteins. For this technique the same electrophoresis system was taken. The compositions of the stacking, spacer and separating gels are shown in the Table 2-7.

Table 2-7 The composition of the solutions used in Tricine-SDS-PAGE

	Stacking gel	Spacer gel	Separating gel
Acrylamide	3.84% (w/v)	9.76% (w/v)	16.5% (w/v)
N, N`methylenbisacrylamide	0.12% (w/v)	0.3% (w/v)	1% (w/v)
SDS	0.1% (w/v)	0.1% (w/v)	0.1% (w/v)
Tris - HCl	1 M pH 8.9	1 M pH 8.9	1 M pH 8.9
Glycerin			13% (v/v)
TEMED	0.05% (v/v)		
APS	0.05% (w/v)		

2D electrophoresis

Two-dimensional electrophoresis was used for the analysis of complex protein mixtures extracted from cells and tissues. This technique sorts proteins according to two independent properties in two discrete steps: the first dimension step, isoelectric focusing, separates proteins according to their isoelectric points; the second dimension step, SDS-PAGE, separates proteins according to differences in their molecular masses [113, 114]. In this way information on protein characteristics such as the pI and the apparent molecular mass could be obtained. The first-dimension separation procedure involves IPG strip rehydration, sample application and isoelectric focusing. For IEF the Multiphor II Electrophoresis system with Immobiline DryStrip gels (IPG) with pH 3-10 and length of 13 cm from Amersham Biosciences was used. The samples were solubilized in the rehydration solution containing 8 M urea, 4 % (w/v) CHAPS, 60 mM DTT, 2 % (v/v) Pharmalyte ^{TM} pH 3-10, 0.002 % (w/v)

bromophenol blue. The solution was applied to the reservoir slots of the Reswelling Tray for the IPG strips rehydration (overnight by room temperature). After that the IPG strips were removed from the tray and positioned in the Immobiline DryStrip aligner for IEF. Table 2-8 shows the parameters used for isoelectric focusing of protein samples.

Table 2-8 Parameters suitable for isoelectric focusing.

Phase	Voltage [V]	Current [mA]	Power [W]	Duration [h:min]
1	300	2	5	0:01
2	3500	2	5	1:30
3	3500	2	5	3:10 – 4:00
Total				4:40 -5:30

After IEF the second dimension separation was performed as described on page 33. For SDS-PAGE the IPG strips were equilibrated in the solution containing 2 % (w/v) SDS, 50 mM Tris-HCl pH 8.8, 30 % (v/v) glycerol, 0.002 % bromophenol blue and 1 % (w/v) DTT.

2.2.5.3 Staining of polyacrylamide gels

Coomassie staining

After electrophoresis the gels were incubated for 15 minutes in color solution (30 % (v/v) ethanol, 10% (v/v) acetic acid, 0.2% (w/v) Coomassie Blue R 250), then washed in distilled water and kept for 20-30 minutes in discoloring solution (30 % (v/v) ethanol, 10% (v/v) acetic acid). Afterwards the colored gels were incubated at least for 3 hours in the drying-fixer solution (5% (v/v) ethanol, 7% (v/v) acetic acid, 10% (v/v) Glycerol). Following this procedure the gels were dried at 65°C in a vacuum using a gel drying system (Biometra D62, Göttingen, Germany).

Silver staining of Rabilloud [115]

After electrophoresis the gels were treated for at least 3 hours with a fixer solution (30 % (v/v) ethanol, 10% (v/v) acetic acid), then washed four times in distilled water, incubated for 1 minute in a sensitizer solution (0.02% (w/v) sodium thiosulfate), then washed once more in distilled water, and afterwards incubated for 60 minutes in silver solution (0.2% (w/v) silver nitrate, 0.03% (v/v) formaldehyde). Following the washing procedure, the gels were developed with the developmental solution (3% (v/v) potassium carbonate, 12 mM sodium

thiosulphate, 0.03% (v/v) formaldehyde). The developing procedure was stopped with a solution containing 30 % (v/v) ethanol and 10% (v/v) acetic acid.

2.2.5.4 Western blotting

The electrophoretically separated proteins were transferred from the polyacrylamide matrix onto a nitrocellulose membrane using a semidry blotting system (Biorad, Germany). The membrane and blot paper were soaked in blotting buffer containing 192 mM glycine, 25 mM Tris base, 20 % (v/v) methanol. The transfer was carried out for 50 minutes at 20V [116].

2.2.5.5 Immunoassays

After blotting the nitrocellulose membrane was washed in PBS buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and then incubated with 10 % Dry Milk in PBS to mask the membrane and avoid unnecessary binding of the primary antibody. The membrane was washed again and afterwards the primary antibody was added to form an antibody-protein complex with the protein of interest. Following the washing step the membrane was incubated in the secondary antibody (against the primary antibody) which was an antibody-enzyme conjugate (HRP) to allow visualizing of the complex with the primary antibody. The blot membrane was then incubated with a reaction mix that was specific for the enzyme. The immobilized proteins were detected using the chemiluminescent detection - ECL system (Amersham, Uppsala, Sweden). In the presence of hydrogen peroxide the horseradish peroxidase (HRP) catalyses the oxidation of luminol. Immediately following the oxidation, the luminol is in an excited state, which decays to the ground state by emitting light. An X - ray film on the blot allowed the detection of a flash of light, which is emitted by the enzyme. The band with a protein of interest appeared wherever there was a protein-primary antibody - secondary antibody-enzyme complex. The exposed X - ray film was developed using a developer and a fixer solution from the firm Tetenal (Nordenstedt, Germany)[117].

2.2.5.6 Cell viability assay

The cell viability in cultures of rat lung epithelial cells, human lung carcinoma epithelial cells and human trachea fibroblasts exposed to arsenicals was examined using the MTT (tetrazolium) assay. This assay monitors conversion of MTT to insoluble purple formazan in the reaction catalyzed by mitochondrial dehydrogenases in viable cells. The rate of the conversion is considered a marker of cell viability. The cells together with the medium were placed into 96 well plates (4000-6000 cells in 100µl per well). MTT was added at the final

concentration of 0.5 mg/ml and the cells were incubated at 37°C for 3 to 4 hours. After incubation the medium was removed and the cells were dissolved in acidic isopropanol. The absorbance of the dye was measured at 570 nm with background subtraction at 630 nm. The results of the MTT assay were confirmed by microscopic observations [118].

2.2.5.7 Size exclusion chromatography

Proteins in the cytosolic fractions from non-labeled and labeled tissues were separated by means of size exclusion chromatography. The Superdex 75PG column and 75PC (Amersham Pharmacia Biotech, Uppsala, Sweden) coupled to the HPLC system HP 1100 (Hewlett Packard/Agilent Technologies, Waldbronn, Germany) were used. The instrumental parameters are shown in the Table 2-9.

After fractionation of the non-labeled cytosolic proteins ICP-MS was employed to determine the trace element-containing proteins on-line in the eluate. The separated fractions of the labeled proteins were measured in the scintillation detector for the determination of the γ-rays of the tracer. After column calibration the native molecular masses of the radiotracer-binding proteins present in the separated fractions could be estimated. It was also the first step of the protein purification.

Table 2-9 Instrumental set up of the chromatographic system

Column	Superdex 75PG (16 x 600 mm)	Superdex 75PC (16 x 600 mm)
Pre-column	Securityguard with GFC-3000 filter inlet	Securityguard with GFC-3000 filter inlet
Eluent	20 mmol l^{-1} Tris buffer, pH 7,4 (HNO_3)	100 mmol l^{-1} Tris buffer, pH 7,4 (HCl)
Flow rate eluent	2,0 ml min^{-1}	2,0 ml min^{-1}
Pressure	Up to 0,3 MPa	Up to 0,3 MPa
Sample volume	500 µl	20 µl
Autosampler temperature	4°C	-

The column was calibrated using a protein standard (Amersham Biosciences, Buckinghamshire, UK) containing Dextran blue (2000 kDa), Albumin (67 kDa), Ovalbumin

(43 kDa) Chymotrypsinogen a (25 kDa), Ribonuclease A (13.7 kDa), Aprotinin (6.5 kDa), Vitamin B_{12} (1.3 kDa).

2.2.5.8 Glutathione peroxidase activity assay

The glutathione peroxidase (Gpx, EC 1.11.1.9) activity was measured using the Glutathione Peroxidase Cellular Activity Assay Kit, purchased from Sigma. This kit uses an indirect determination method. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by Gpx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP is indicative of the Gpx activity, since Gpx is the rate-limiting factor of the coupled reactions [119, 120]. The reaction is schematically shown below:

$$ROOH + 2\,GSH \xrightarrow{Gpx} ROH + H_2O + GSSG$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+$$

The samples were diluted in 50 mM Tris-HCl buffer pH 8.0 to get a protein concentration of 50µg in 1 ml. 50 µl of NADPH and 10 µl of the sample were added to the cuvet filled already with the appropriate volume of Tris buffer and mixed by inversion. The total volume in the cuvet was 1 ml. The reaction was performed at 25 °C and at pH 8.0, and was started by adding 10µl tert-butyl hydroperoxide. This peroxide reacts only with the selenium - containing glutathione peroxidase. The final concentrations of the reagents in the assay mixture were: 0.25 mM NADPH and 300 µM tert-butyl hydroperoxide. A blank measurement was performed using a Tris buffer instead of the enzyme. The amount of the enzyme in the sample was calculated as described elsewhere [120].

2.2.5.9 MALDI-MS

The application of mass spectrometry to large complex molecules like proteins has been made possible by the development of novel ionization techniques which enable large molecules (>200 kDa) to be introduced into the mass spectrometer in an intact form suitable for analysis [121]. Of the various techniques that have been developed, matrix-assisted laser desorption ionization (MALDI) is one of the best-suited for macromolecules. In MALDI, the sample is

embedded in a non-volatile matrix such as nicotinic acid or 2, 5-dihydroxybenzoic acid. The sample matrix is introduced into the mass spectrometer, charged to the high-voltage and exposed to the high-energy laser beam. The matrix material is chosen to absorb the laser radiation and the radiation causes the matrix and the sample molecules to vaporize or 'sputter' into the gas phase [122]. A typical protocol consists of the following steps: separation of proteins from a given biological sample by gel electrophoresis (2D-PAGE), excision of spots from the gel and digestion by trypsin, and extraction from the gel and analysis by mass spectrometry. The protein identification is achieved by mass fingerprint of peptides derived from proteins in databases. The analysis has been carried out by the firm Wita (Berlin, Germany).

2.2.6 Tissue preparation

2.2.6.1 Homogenization of tissues and cells

The tissues of lung and trachea were homogenized using an isotonic medium containing 0.25 M sucrose and 1 mM EDTA buffered with a suitable organic buffer: Tris or Hepes at pH 7.0-7.6. The content of the medium used for homogenization depends on the isolated organelles. Specific media tailored to the requirements of specific organelles are shown in the Table 2-10. The tissue was first finely chopped with scissors and then destructed using a mechanical shear, Potter-Elvehjem or sonification. Tissue and cell debris was removed by low speed centrifugation (200×g) [123].

Table 2-10 Homogenization media for lung and trachea organelles.

Organelles	Homogenization medium
Nuclei	0.25 M sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4
Mitochondria	0.225 M mannitol, 0.075 M sucrose, 0,5 mM EGTA, 2 mM MOPS pH 7.4
Endoplasmic reticulum	200 mM mannitol , 50 mM sucrose, 1 mM EDTA, 10 mM hapes-NaOH pH 7.4
Golgi membranes	0.25 M sucrose, 10 mM Hepes-NaOH pH 7.6, 1 mM EDTA
Ribosomes	0.25 M sucrose, 5 mM $MgCl_2$, 100 mM KCl, 0.2 mM EDTA, 20 mM DTT, 50 mM Tris-HCl pH 7.5
Lysosomes	0.25 M sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4

The tissue culture cells were homogenized using a lysis buffer containing 150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl flouride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate, 5 µg/ml of aprotinin, 5 µg/ml of leupeptin) and then sonicated.

2.2.6.2 Isolation of subcellular fractions

2.2.6.2.1 Separation by size: differential centrifugation

Differential pelleting was applied for separation of the homogenate components into: nuclei, mitochondria, microsomes, and the soluble portion of the cell, the cytosol, by differential centrifugation at 1000 ×g for 10 min, 10000 ×g for 30 min and 100000 ×g for 110 min (Optima MAX with MLA-55 rotor, Beckman Instruments GmbH, Munich, Germany) [123]. As the fractions differ significantly in size they also differ in their rate of sedimentation and can be separated by centrifugation. All these operations were carried out at 4°C. The pellets were washed by resuspending in homogenization medium and recentrifuged under the same conditions to minimize the contaminations. Hence differential pelleting, as this technique is termed, is highly effective in separating particles into broad size classes but not suitable for separating particles which are similar in size.

2.2.6.2.2 Separation by density

Separation by density was applied for the isolation of nuclei, mitochondria, rough and smooth endoplasmic reticulum, Golgi membrane, lysosomes and ribosomes. The separation of organelles on the basis of their density was carried out by layering the organelles over or under a density gradient and centrifuging to equilibrium.

2.2.6.2.3 Purification of nuclei - method of Windell and Tata [124]

The tissue was homogenized in 9 volume of the cold homogenizing medium. The homogenate was filtered through four layers of muslin and centrifuged at 600×g for 10 minutes at 5°C. The pellet was resuspended in half the original volume of the homogenizing medium and centrifuged again as described above. A pellet of the crude nuclei was resuspended and homogenized in the 2.2 M sucrose solution containing 1 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4 to remove membrane contamination. The suspension of nuclei was centrifuged at 80000×g for 80 minutes at 5°C in a swinging-bucket rotor. The cream-colored pellet appearing after centrifugation was resuspended in the homogenization medium. To remove the nuclear

membrane the nuclei pellet was washed with the homogenizing medium containing 0.5 % Triton X-100.

2.2.6.2.4 Purification of mitochondria - method of Chance and Hagihara

The tissues were homogenized with 2 volumes of the homogenization medium. The homogenate was centrifuged at 800×g for 7 minutes to remove nuclei, cell debris and red blood cells. After that the supernatant was carefully decanted and recentrifuged at 800×g for 7 minutes. The new supernatant was centrifuged again at 6500×g for 15 minutes to sediment a crude mitochondrial pellet. The mitochondrial pellet was resuspended in a small volume of homogenization medium and disaggregated by hand homogenization. The suspension was recentrifuged at 6500×g for 7 minutes. The final mitochondrial pellet was resuspended in the homogenization buffer [125].

2.2.6.2.5 Purification of rough and smooth endoplasmic reticulum - method of Bergstrand and Dallner

The tissue was homogenized with three volumes of the homogenization medium and then centrifuged at 1000×g for 10 minutes. The supernatant 1 was centrifuged at 3000×g for 10 minutes, and then the supernatant 2 was centrifuged at 10000×g for 30 minutes. The post-mitochondrial supernatant was layered on the top of polycarbonate tubes filled with 3 ml of 1.3 M sucrose-Tris-$CsCl_2$ and 1.5 ml of 0,6 M sucrose–Tris-$CsCl_2$. This gradient was centrifuged at 100000×g for 90 minutes using a fixed-angle rotor. After that two major fractions were obtained: a double layered band at or just below the 0.6-1.3 M sucrose interface - smooth endoplasmic reticulum and the pellet which contained the rough endoplasmic reticulum. To remove the rest of the material from the 1.5 M sucrose layer the suspension was diluted with 3 volumes of 5 mM Tris-HCl pH 8 and pelleted by centrifugation at 160000×g for 30 minutes. Both pellets were resuspended in 0.25 M sucrose, 5 mM Tris-HCl pH 8.

2.2.6.2.6 Purification of ribosomes

The tissues were homogenized in the homogenization medium (2 ml per 1 g tissue). The nuclei and mitochondria were removed by centrifugation at 12000×g for 20 minutes. The upper 2/3 of the supernatant was carefully collected and filtered through four layers of cheesecloth. To release the ribosomes from the microsomal membranes, freshly prepared 10% sodium deoxycholate was added. The post-mitochondrial supernatant was layered over the sucrose pad (consisting of 1 M sucrose in 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM

KCl, and 10 mM DTT) and centrifuged overnight at 130000×g. After centrifugation the supernatant was carefully removed and the clear ribosomal pellet was rinsed with a buffer containing 5 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM DTT and then resuspended gently in the same buffer. A freshly prepared puromycin solution was added and the solution was incubated 30 minutes on ice and 15 minutes at 37°C. The solution was clarified by centrifugation for 5 min at 10000×g.

2.2.6.2.7 Purification of lysosomes – method of Maunsbach

The tissue was homogenized in homogenization medium. The homogenate was centrifuged for 10 minutes at 1000×g. The supernatant obtained was centrifuged at 3000×g for 10 minutes, and then this second supernatant again at 10000×g for 20 minutes. The lighter material from the pellet was removed by resuspending gently in a solution containing 0.3 m sucrose, 5 mM Tris-HCl and 1 mM EDTA. The remaining dark-brown lysosomal pellet was resuspended in the same solution. The suspension was loaded on the top of a sucrose gradient consisting of following sucrose solutions 2.1 M, 1.7 M, 1.4 M, and 1.1 M. The gradient was centrifuged at 95000×g for 4 hours in a swinging rotor.

2.2.6.3 Identification of separated materials

The organelles were identified either by examination under the electron microscope or by biochemical techniques.

2.2.7 Text and Data converting

Text processing was carried out using a *Microsoft World 2002*. The data, tables, figures and photos were dealt with either by using *Microsoft Excel 2002* or *Adobe Photoshop*. The autoradiograms were evaluated using the software *Aida 2.4.3*.