6. Tracer experiments with ^{73}As

In a first series of experiments the effects of the selenium status on the distribution of ^{73}As among the body compartments were analyzed. Here selenium-deficient and selenium-sufficient rats were labeled *in vivo* with ^{73}As administrated either orally or ip. For the labeling experiments male animals were used (for details see section 2.1.3).
In the second part of experiments the distribution of arsenic among the subcellular fractions of several tissues and of cultured cells was investigated. The tissues were homogenized and partitioned into subcellular fractions by differential centrifugation as described in section 2.2.4.2. The proteins present in those samples were separated by SDS-PAGE or 2D electrophoresis, and the labeled compounds detected autoradiographically in the gel.

6.1 Distribution of ^{73}As in the tissues of the rat

Wistar rats (n=2) fed either a selenium-sufficient or a selenium-deficient diet were labeled *in vivo* by injection of 6 MBq ^{73}As (130 ng arsenic) i.p per animal. The tracer activities present in the animals were measured after 24 and 48 hours. After 24 hours the selenium-deficient animals still contained 95% of the original dose, while in the selenium-sufficient rats only 86% were found. After 48 hour the selenium-deficient animals had retained 89.1% of the radioactive arsenic dose and the selenium-sufficient 25%. In a second experiment ^{73}As was administered orally (n=3). After 48 h 88.9% and 70.6% of ^{73}As remained in the selenium-deficient animals and selenium-sufficient animals, respectively.
The animals were killed 48 hours after the arsenic administration, and the tissues were taken for the tracer measurement. The Figure 6-1 shows the distribution of ^{73}As in the different tissues.

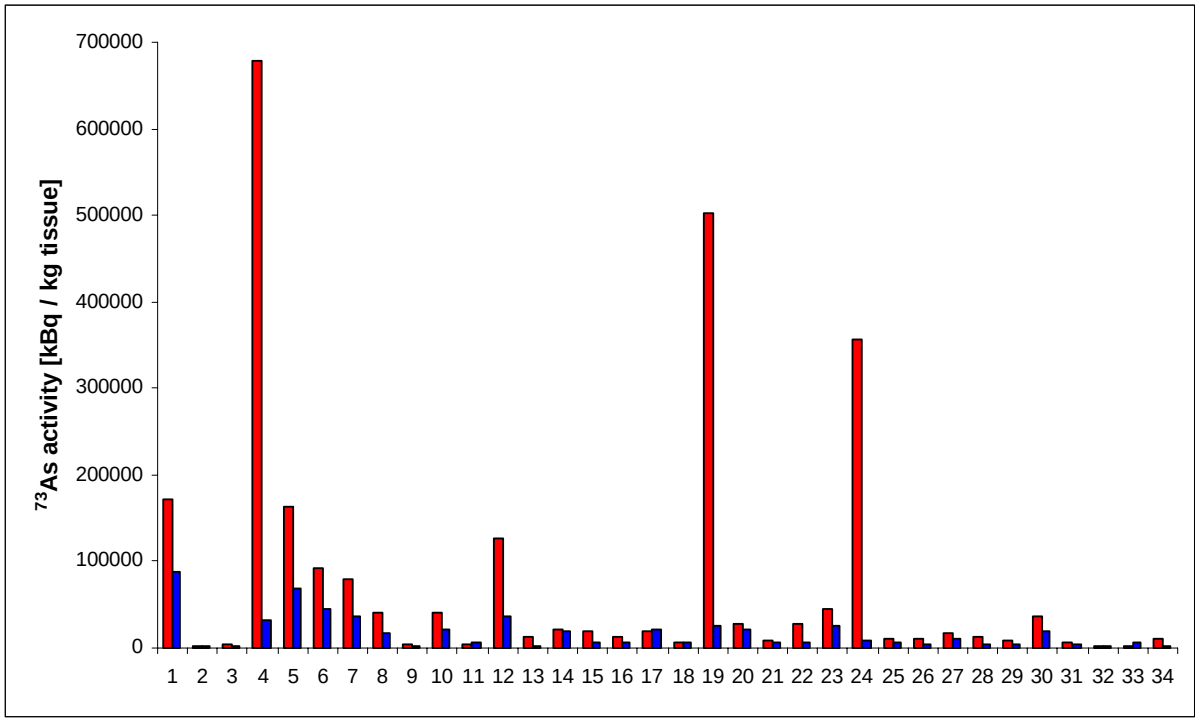

Figure 6-1 Distribution of ^{73}As in the tissues (wet mass) of rats (n=2) fed either a selenium-deficient (red bar) or a selenium-sufficient diet (blue bar) 48 hours after injection of the tracer i.p.; blood - 1, skin - 2, ear lobule - 3, pancreas - 4, spleen - 5, liver - 6, adrenal gland - 7, kidney - 8, prostate - 9, epididymis - 10, testis - 11, spermatic ducts (ductus deferens) - 12, seminal vesicles - 13, small intestine - 14, colon - 15, duodenum - 16, stomach -17, brown adipose tissue -18, diaphragm - 19, heart - 20, gullet - 21, trachea - 22, lung - 23, thymus - 24, sternum - 25, muscle - 26, thyroid -27, tongue - 28, brain - 29, pituitary - 30, spinal marrow - 31, eye - 32, plasma - 33, nail – 34.

Remarkable differences in ^{73}As distribution were observed between selenium-deficient and selenium-sufficient tissues, with a much higher percentage of the tracer dose being retained in the tissue of the selenium-deficient diet animals. The tissues vary in the ^{73}As content. In the tissue of the selenium-deficient animals the tracer content decreased in the following order: pancreas> diaphragm> thymus> blood> spleen> spermatic ducts> liver> adrenal gland> lung> epididymis > kidney> pituitary> heart, trachea> small intestine, colon, duodenum, stomach, tongue, thyroid > brown adipose tissue, gullet, sternum, muscle, brain, spinal marrow, nail, seminal vesicles > ear lobule, prostate, testis> skin, eye, plasma.

In the tissues of selenium sufficient animals obviously less radioactive arsenic was found. The distribution pattern of the tracer among the body comportments differed from that of selenium-deficient animals. Here the tracer content decreased in the fallowing way: blood>

spleen> liver> adrenal gland> pancreas> spermatic ducts> epididymis, small intestine, stomach, lung> heart, pituitary> kidney> colon, gullet, thymus, sternum, thyroid> testis, duodenal, brown adipose tissue, trachea, muscle, tongue, brain, nail> ear lobule, prostate, seminal vesicles, spinal narrow> skin, eye, plasma.

For each tissue a retention factor of ^{73}As was calculated by relating its specific activity in the deficient rats to the corresponding value in the sufficiently supplied animals. These retention factors are shown in the Figure 6-2.

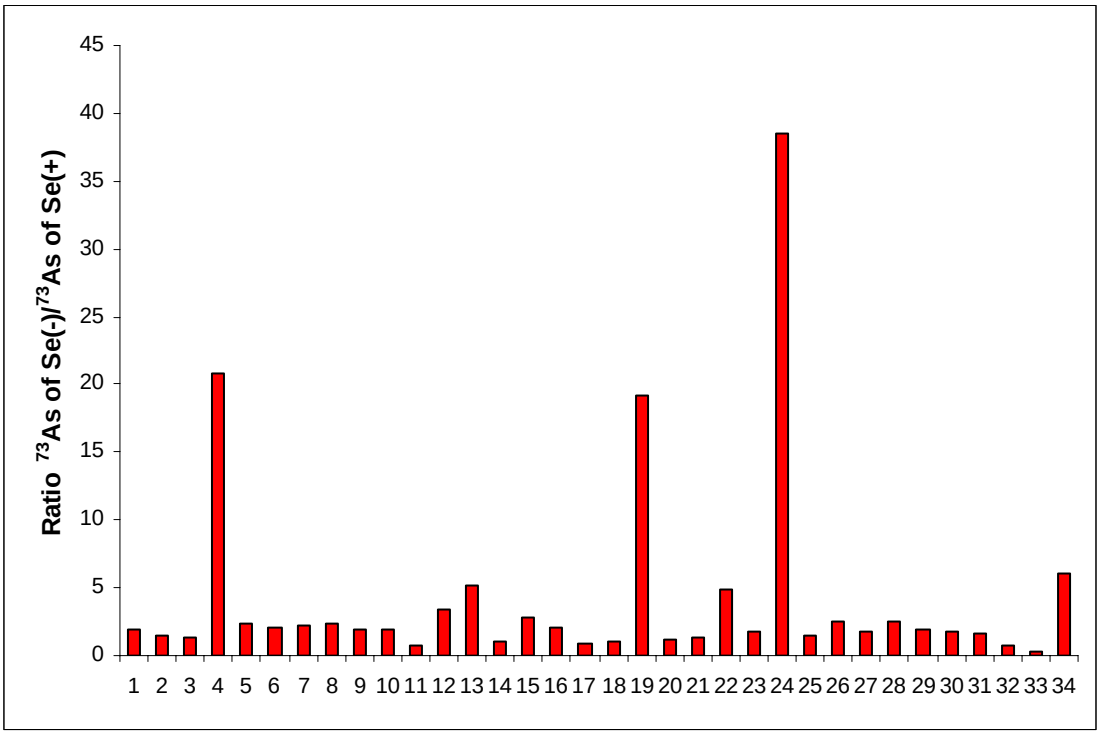

Figure 6-2 Retention factor of ^{73}As in the tissues of selenium-deficient and selenium-sufficient animals 48 hours after administration of the tracer i.p.; blood - 1, skin - 2, ear lobule - 3, pancreas - 4, spleen - 5, liver - 6, adrenal gland - 7, kidney - 8, prostate - 9, epididymis - 10, testis - 11, spermatic ducts (ductus deferens) - 12, seminal vesicles - 13, small intestine - 14, colon - 15, duodenum - 16, stomach - 17, brown adipose tissue -18, diaphragm - 19, heart - 20, gullet - 21, trachea - 22, lung - 23, thymus - 24, sternum - 25, muscle - 26, thyroid -27, tongue - 28, brain - 29, pituitary - 30, spinal marrow - 31, eye - 32, plasma - 33, nail – 34.

The data show that after administration i.p. of a small amount of ^{73}As in the selenium-deficient animals the element is preferentially taken up by the thymus, pancreas and diaphragm.

The distribution pattern of arsenic among the body compartments after oral administration is shown in the Figure 6-3. For these data, the retention factors have been calculated. They are presented in the Figure 6-4.

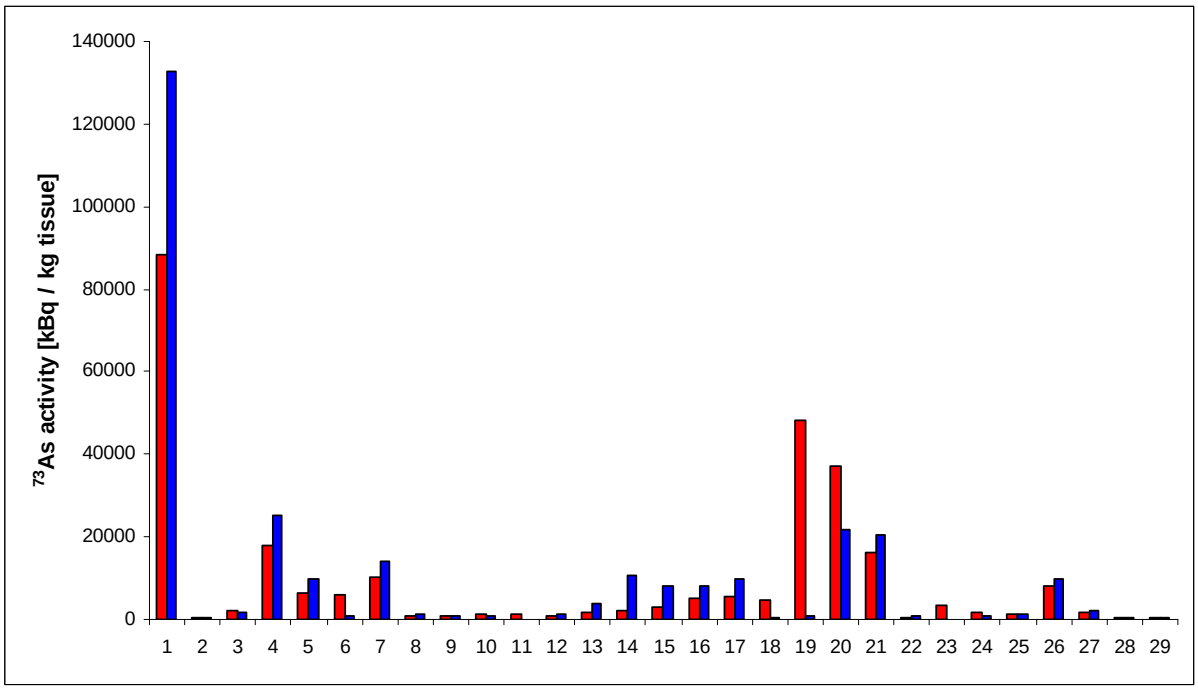

Figure 6-3 Distribution of ^{73}As in the tissues (wet weight) from rats fed either a selenium-deficient (red bar) or a selenium-sufficient diet (blue bar) 48 hours after oral administration of the tracer; blood - 1, skin - 2, pancreas - 3, spleen - 4, liver - 5, adrenal gland - 6, kidney - 7, prostate - 8, epididymis - 9, testis - 10, spermatic ducts (ductus deferens) - 11, seminal vesicles - 12, small intestine - 13, colon - 14, duodenum - 15, diaphragm - 16, heart - 17, gullet - 18, trachea - 19, lung - 20, thymus - 21, muscle - 22, thyroid -23, tongue - 24, brain - 25, pituitary - 26, spinal marrow - 27, eye - 22, plasma - 29

The distribution pattern of the orally administered arsenic in the rat tissues differs from that after i.p. injection. The tracer is mostly present in the blood of the selenium-sufficient and selenium-deficient animals. In the trachea, gullet, lung, thyroid, adrenal gland and spermatic ducts the arsenic concentration was higher in the selenium-deficient animals, in contrast in the small intestine, colon, kidney, liver, spleen, heart, diaphragm and duodenum much more tracer was found in the selenium-sufficient animals. With the other tissues not significant differences between those two groups were observed.

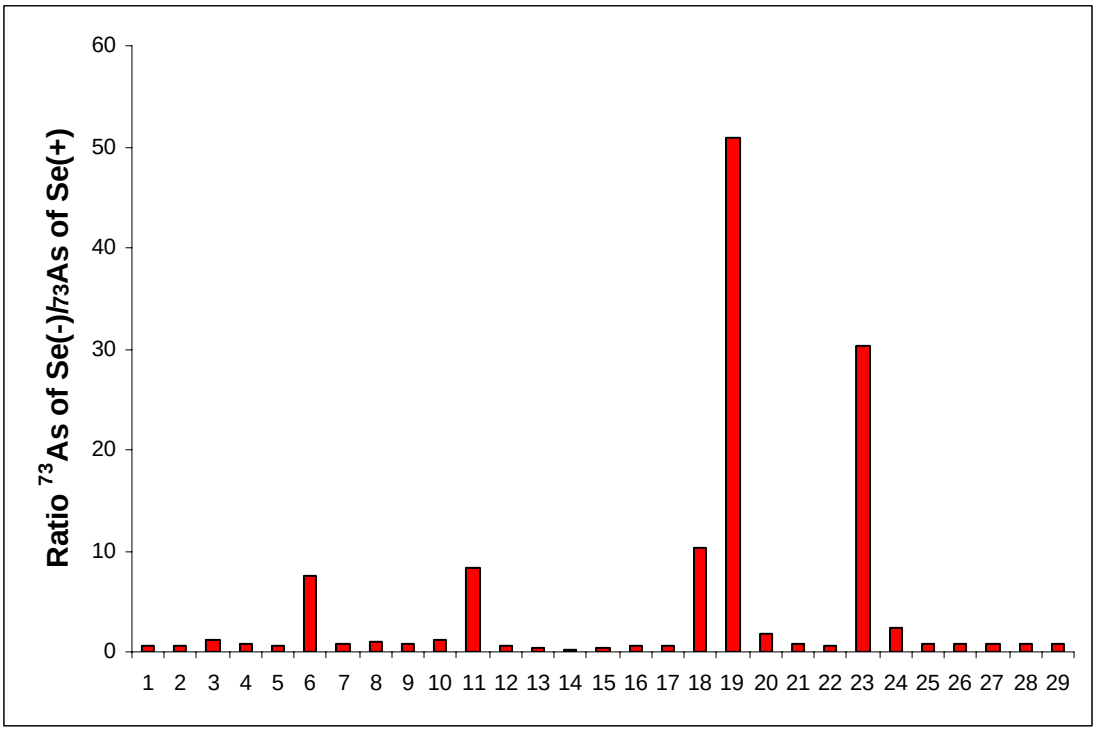

Figure 6-4 Retention factor of ^{73}As in the tissues of selenium deficient and selenium sufficient animals after oral administration of the tracer; blood - 1, skin - 2, pancreas - 3, spleen - 4, liver - 5, adrenal gland - 6, kidney - 7, prostate - 8, epididymis - 9, testis - 10, spermatic ducts (ductus deferens) - 11, seminal vesicles - 12, small intestine - 13, colon - 14, duodenum - 15, diaphragm - 16, heart - 17, gullet - 18, trachea - 19, lung - 20, thymus - 21, muscle - 22, thyroid - 23, tongue - 24, brain - 25, pituitary - 26, spinal marrow - 27, eye - 22, plasma – 29

6.2 Distribution of ^{73}As among subcellular fractions

In order to obtain some information on the sites of action of arsenic and thus on its possible functions, the subcellular distribution of the tracer was investigated. The tissues with the highest arsenic retention after administration of the tracer i.p. were taken for this investigation. Pancreas, thymus, spleen, liver, diaphragm, and lung were homogenized and than partitioned by differential centrifugation into nuclear, mitochondrial, microsomal and cytosolic fractions. The protein concentration and the activity of the tracer were determined in all fractions. After that, the ^{73}As activity was related to the protein content of each sample. The results are shown in the Figures 6-5 and 6-6.

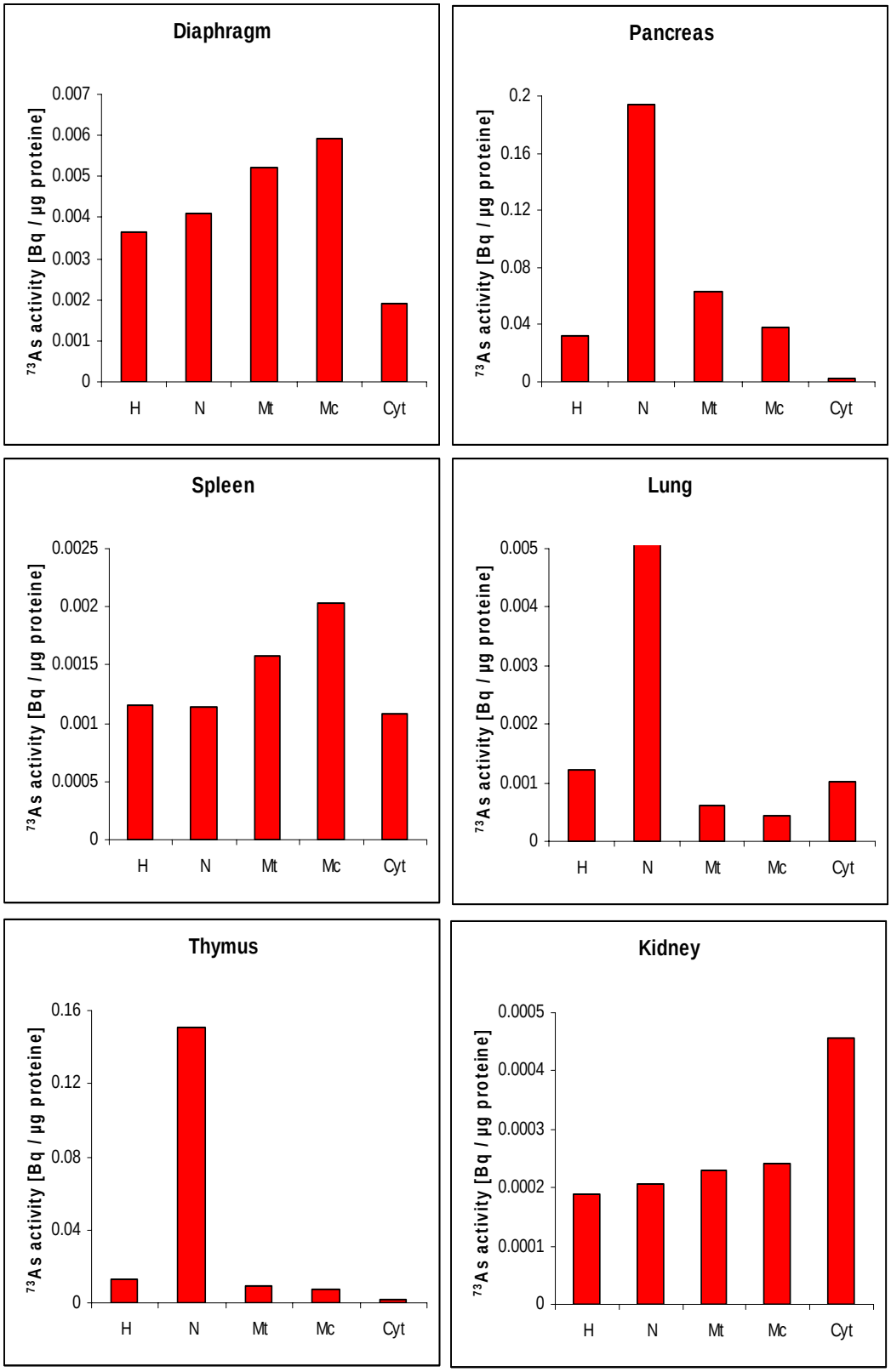

Figure 6-5 Distribution of ^{73}As in the homogenates and subcellular fractions of the diaphragm, pancreas, spleen, lung and thymus of selenium-deficient animals after administration of the tracer i.p.; H- homogenate, N-nuclei, Mt- mitochondria, Mc- microsome, Cyt- cytosol

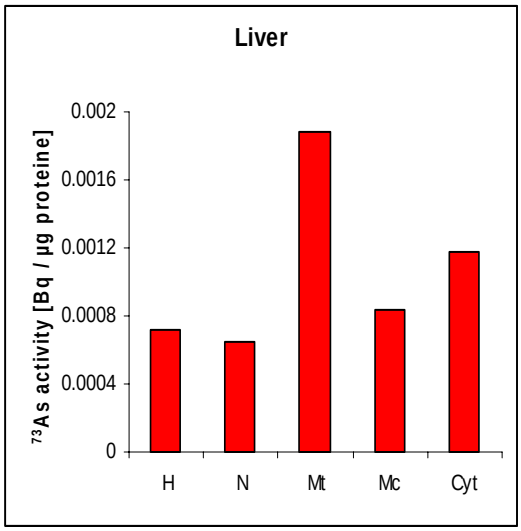

Figure 6-6 Distribution of ^{73}As in the homogenates and subcellular fractions of the liver of selenium-deficient animals after administration of the tracer i.p.; H- homogenate, N-nuclei, Mt- mitochondria, Mc-microsome, Cyt- cytosol

In the homogenates and subcellular fractions of these tissues differences in the ^{73}As distribution were observed. The retention of the tracer was highest in the cellular fraction of the thymus and pancreas, whereas in the cellular compartments of the liver, lung and diaphragm the retention was similar. The lowest tracer activity was found in the kidney compartments.

In the pancreas, lung and thymus samples the highest specific activity was found in the nuclear fraction. In the diaphragm and spleen the tracer was retained mainly in the microsomes, and in the liver in the mitochondria. The liver and spleen nuclei contained the lowest amount of the tracer. In the spleen the lowest ^{73}As content was found in the cytosolic fraction. The retention of the tracer in the nuclei, mitochondria and microsomes of the kidney was similar.

The tracer was found to be incorporated differently within the different cell compartments of the different tissues. It was mainly found in the nuclei, but also in the cytosolic and mitochondrial fraction. The highest arsenic concentration in the cytosolic kidney fraction may indicate that in this organ (responsible for excretion of many metabolites) arsenic is present in the form of soluble compounds.

6.3 Arsenic-binding proteins in the rat tissues

In the following experiment the distribution of the arsenic-containing proteins in the homogenates of several rat tissues was studied. For these experiments only selenium-deficient animals had been taken, because of the higher retention of the tracer in these animals. The spleen, adrenal gland, spermatic ducts, diaphragm, liver, thymus, trachea, brain, heart, lung, pancreas, testis, epididymis, small intestine and kidney were homogenized. The proteins present in these tissues were separated by means of SDS-PAGE and the arsenic-containing proteins then identified by autoradiography. The autoradiograms are shown in the Figure 6-7.

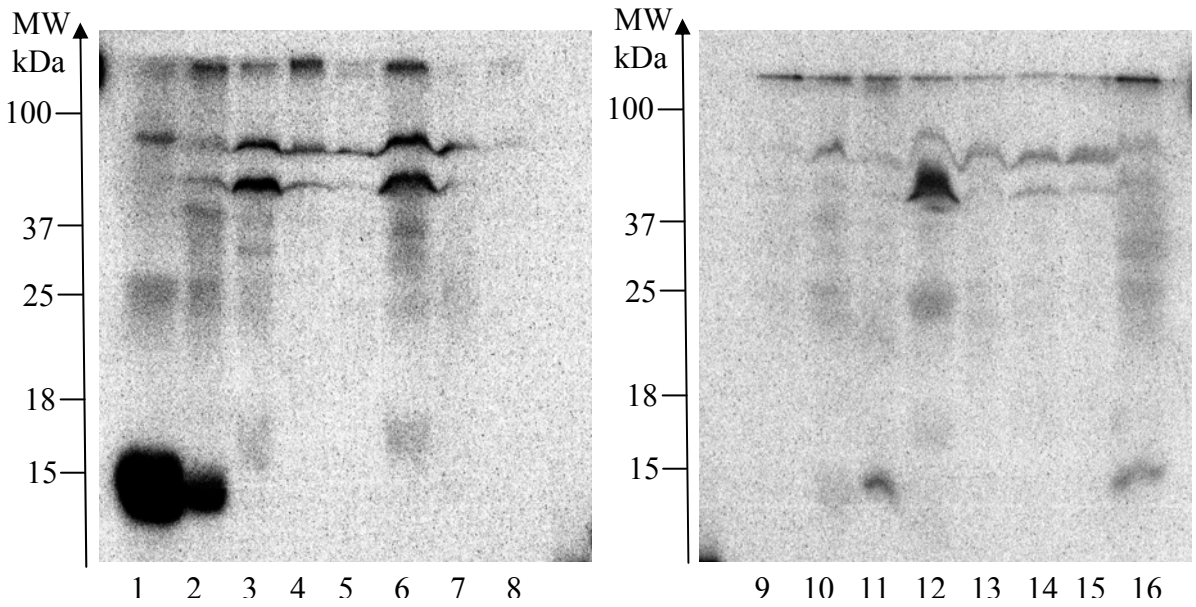

Figure 6-7 Autoradiogram of the ^{73}As-labeled proteins in the blood -1 and homogenates of spleen- 2, adrenal gland- 3, spermatic ducts- 4, diaphragm- 5, liver- 6, thymus- 7, trachea- 8, brain- 9, heart- 10, lung- 11, pancreas -12, testis -13, epididymis -14, small intestine- 15 and kidney- 16 after SDS-PAGE

The evaluation of the autoradiograms shows that there were several arsenic-binding proteins present in the analyzed tissues. The proteins labeled with ^{73}As had relative molecular masses of >250, 75, 50, 37, 29 – 30, 25, 16, and 15 kDa. The ^{73}As-binding bands with molecular masses of >250, 75, and 50 kDa were detected in all homogenates. The band at 37 kDa was present in the blood, spleen, adrenal gland, liver, thymus, heart, kidney and pancreas. The homogenates of the adrenal gland, kidney, and thymus had labeled bands at 30 -29 kDa. The band at 25 kDa was found in the blood and in the homogenates of the spleen, thymus, heart, kidney, and pancreas. In the homogenates of pancreas, thymus, spleen and adrenal gland a

further ^{73}As-containing protein was detected at 16 kDa. The very strongly labeled band at 15 kDa was found in the blood and in the homogenates of the spleen, heart and kidney.

6.3.1 Subcellular distribution of the arsenic-binding proteins

The homogenates of the thymus, spleen, pancreas, diaphragm, lung and liver were fractionated into the nuclear, mitochondrial, microsomal and cytosolic fraction. The proteins present in those fractions were separated by SDS PAGE and then the arsenic-containing proteins were identified autoradiographically via the ^{73}As tracer. The autoradiograms in the Figures 6-8 – 6-9 show the arsenic-containing proteins in the subcellular fractions of the thymus, spleen, pancreas, diaphragm, lung and liver.

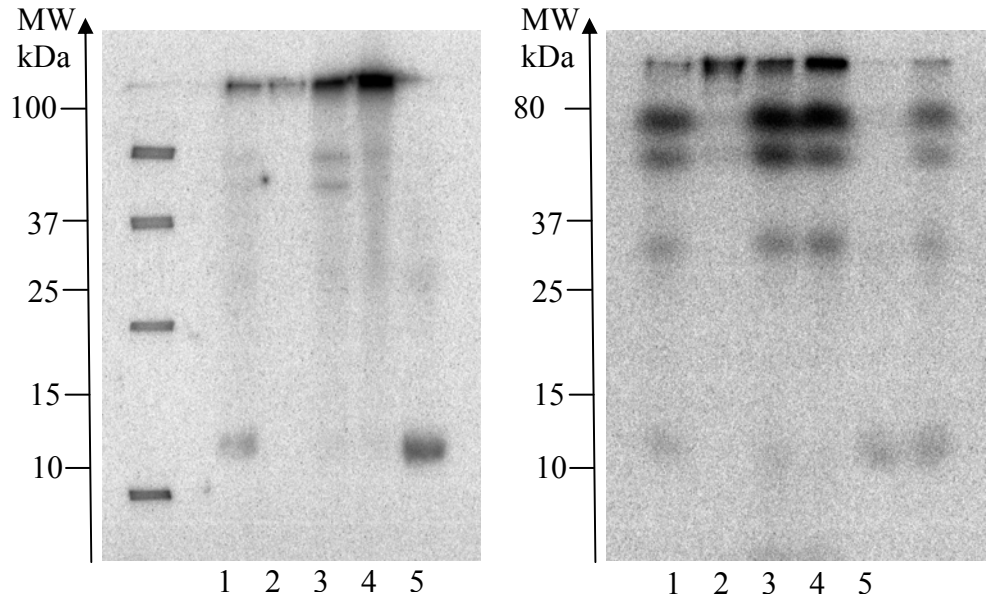

Figure 6-8 Autoradiogram of the ^{73}As-labeled proteins in homogenates and subcellular fractions of the spleen (left) and thymus (right) after SDS-PAGE: 1– homogenate, 2- nuclei, 3- mitochondria, 4- microsomes, 5- cytosol

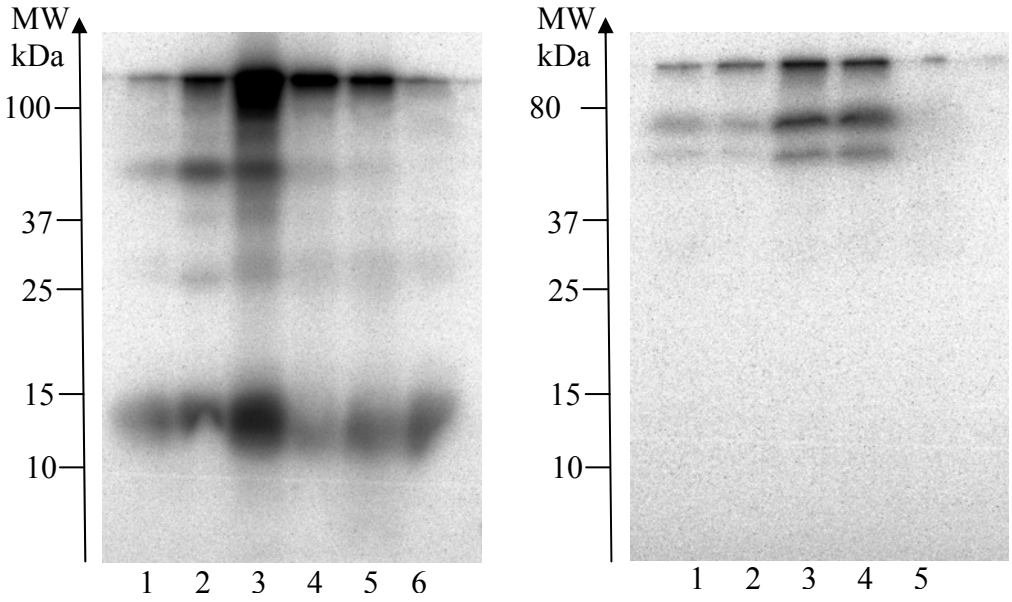

Figure 6-7 Autoradiogram of the ^{73}As-labeled proteins in homogenates and subcellular fractions of the pancreas (left) and diaphragm (right) after SDS-PAGE: 1– homogenate, 2- nuclei, 3- mitochondria, 4- microsomes, 5- cytosol

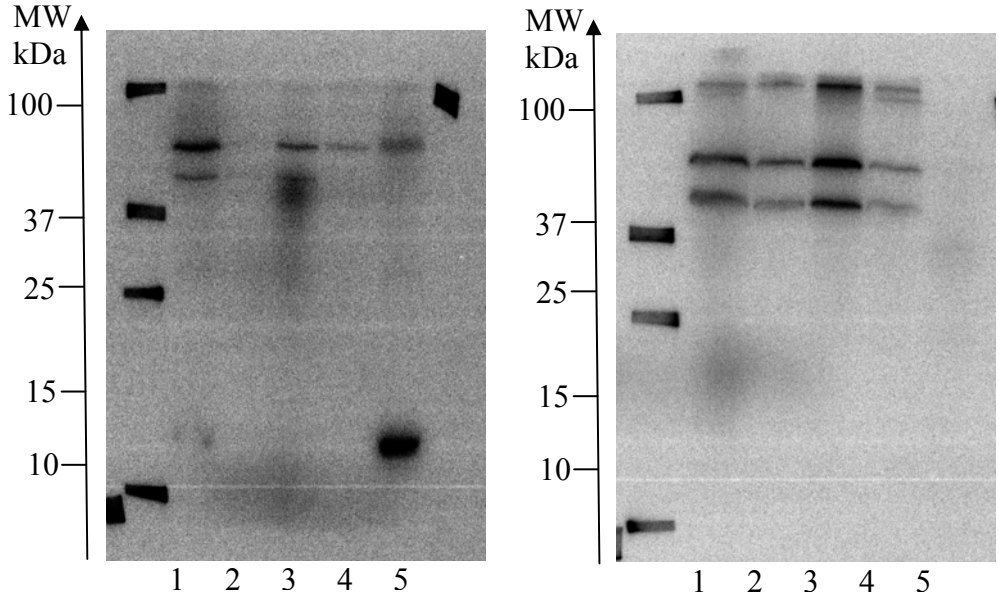

Figure 6-9 Autoradiogram of the ^{73}As-labeled proteins in homogenates and subcellular fractions of the lung (left) and liver (right) after SDS-PAGE: 1– homogenate, 2- nuclei, 3- mitochondria, 4- microsomes, 5- cytosol

The aim of these studies was the comparison of the distribution pattern of the arsenic-binding proteins among the cellular compartments of different tissues of the rat. There were remarkable differences observed in the characteristic of the arsenic-containing proteins between the tissues after SDS-PAGE.

In every fraction of the spleen a band with the molecular mass of > 250 kDa was found. It was most strongly labeled in the mitochondrial and microsomal fractions. The bands at 75 kDa and 50 kDa were detected in the homogenate, mitochondria and microsomes. A weakly labeled band at 30 kDa could be seen in the homogenate, mitochondria and cytosol. In the homogenate and cytosol a quite strongly labeled band at 15 kDa was observed.

In the thymus a band at > 250 kDa was detected. It was most strongly labeled in the microsomes and mitochondria. The bands at 75 kDa, 50 kDa and 37 kDa were found in the homogenate, mitochondria and microsomes. The first two bands were strongly labeled in the mitochondria and microsomes. The 15 kDa band was only present in the homogenate and cytosol.

In the subcellular compartments of the pancreas bands at > 250 kDa, 50 kDa, 37 kDa, 30 kDa, 25 kDa and 15 kDa were detected. For all bands the highest tracer activity was found in the mitochondrial fraction. The ^{73}As-containig band found at 15 kDa was predominantly labeled in all fractions.

The cellular compartments of the diaphragm contained ^{73}As-labeled proteins at > 250 kDa, 75 kDa, and 50 kDa. The last two were missing in the cytosolic fraction.

The subcellular fractions of the lung contained the following arsenic-binding proteins: > 250 kDa, 75 kDa and 50 kDa. The bands at 75 kDa and 50 kDa were most strongly labeled in the mitochondria. There was a weakly labeled band at 25 kDa in the homogenate, mitochondria and cytosol. In the cytosolic fraction a strongly labeled band at 15 kDa was observed.

In the homogenate, nuclei, mitochondria and microsomes of the liver two labeled bands at >250 kDa, and one each at 75 kDa and at 50 kDa were found, while in the cytosol only one weakly labeled protein at 37 kDa was observed. In the homogenate a diffuse band at 15 kDa was detected. All those proteins were most strongly labeled in the mitochondrial fraction.

The differences found in the distribution of the arsenic-binding proteins among the tissues and their cellular compartments suggest that the arsenic compounds may be involved in different intracellular processes.

6.4 Cell culture experiments

Two cell lines, A549 human epithelial cells and CCL rat epithelial cells, were labeled in vitro with ^{73}As. For this experiment 185.7 kBq (0.0039 µg of arsenic) were added per flask filled with 20 ml of the medium. The final arsenic concentration in the medium was 2.7 µM. After 24 h cells and medium were separated. The cells were homogenized by sonification with 20 mM Tris-HCl pH 7.4. The homogenate was centrifuged at 100 000×g for the isolation of the cytosolic fraction. After determination of the protein concentration and the activity in all fractions obtained, the samples were prepared for SDS-PAGE and 2D-electrophoresis.

6.4.1 ^{73}As activity in the cell compartments

In the homogenates, pellets (100000×g) and cytosols of the lung epithelial cells from rat and human, the ^{73}As activity was determined and related to the protein content or volume of each of the samples. The results for the specific tracer activity obtained in this way are shown in the Figure 6-10.

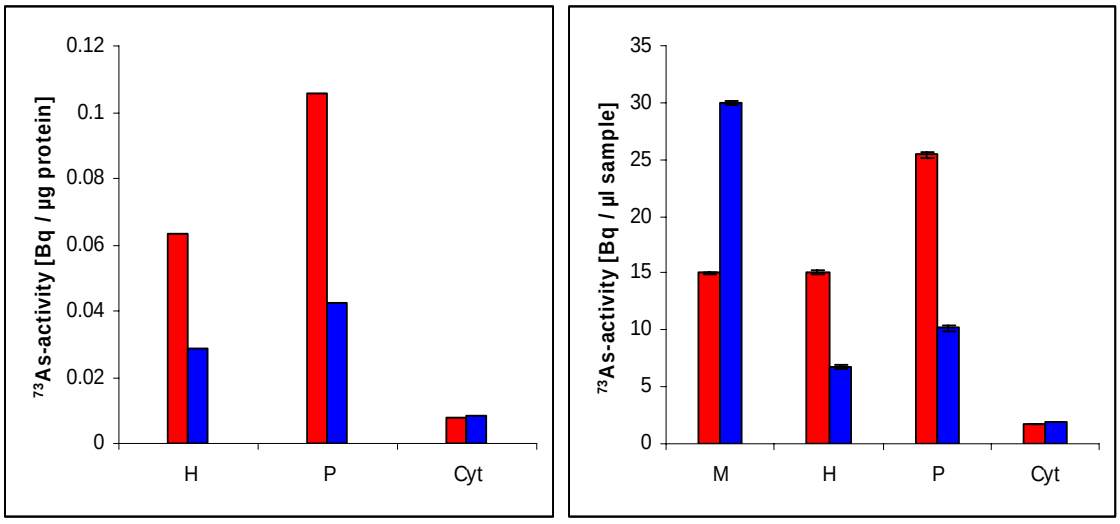

Figure 6-10 Distribution of ^{73}As in the homogenate - H, pellet 100000×g – P, cytosol – Cyt, and medium –M of human epithelial cells (red bar) and rat epithelial cells (blue bar) n=3 calculated per µg of protein (left figure) and per µl of the samples (right figure)

In the homogenates and pellets of the human and rat lung epithelial cells significant differences in the ^{73}As distribution were observed. In both cell types the highest specific

activity was found in the pellets. The content of the tracer was significantly higher in the pellets of the human cells. The lowest level of ^{73}As was in the cytosolic fractions of both groups. Interestingly, quite a large amount of the tracer remained in the medium of the rat cells. This finding may have two explanations. Either the cells are able to metabolize and excrete arsenic very fast or the metabolism of arsenic in these cells is much slower. It was therefore necessary to identify the form of arsenic in the medium. Accordingly some experiments have been carried out. They are described in section 6.4.3.

6.4.2 Arsenic-binding proteins in the cells of the respiratory tract

The proteins present in the homogenates, pellets and cytosolic fractions of the cells were separated by means of SDS PAGE and the arsenic-containing proteins then identified by autoradiography. The autoradiograms are shown in the Figure 6-11. In these samples six proteins containing ^{73}As have been found. Their relative molecular masses were: >250 kDa, 75 kDa, 50 kDa, 37 kDa and 18 kDa.

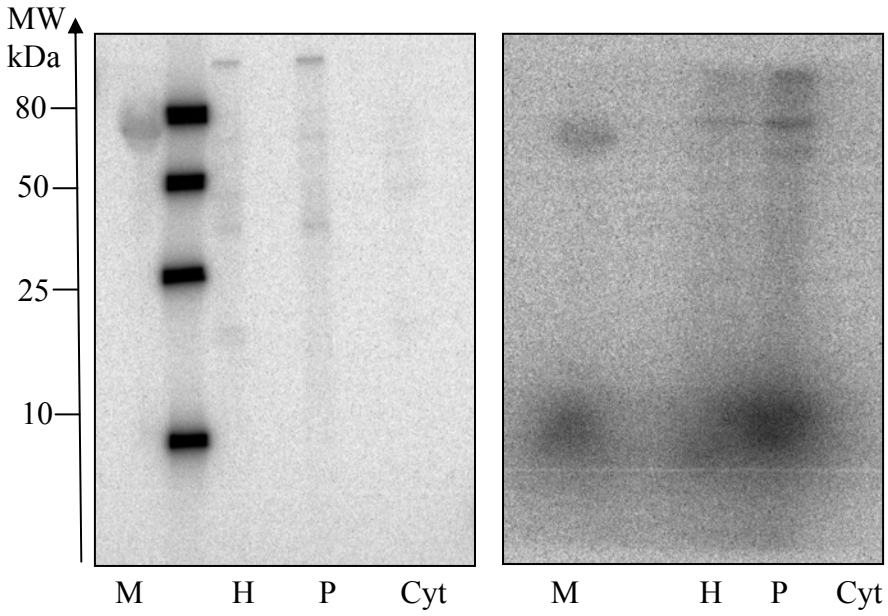

Figure 6-11 Autoradiogram of the ^{73}As-labeled proteins in the medium (M), homogenate (H), pellet 100000×g (P) and cytosolic fractions (Cyt) of the rat lung epithelial cells (left) and human epithelial cells (right) after SDS-PAGE

In the media from both cell types one ^{73}As-containing band with a molecular mass of about 75 kDa was detected. In the homogenate of the CCL cells six bands at > 250 kDa, 75 kDa, 50 kDa, 37 kDa and 18 kDa could be seen, while in the pellet only bands at > 250 kDa, 75 kDa,

50 kDa and 37 kDa were detected. In the cytosols two weakly labeled bands at about 50 kDa and 18 kDa were present. The homogenate and pellet of the A549 cells contained four radioactive bands at > 250 kDa, 75 kDa, 50 kDa and one very blurred at about 12 kDa, whereas in the cytosols the tracer concentration was too low to detect ^{73}As-labeled bands.

SDS PAGE is a denaturing separation method and the proteins lose their native structure. Most elements which are bound non-covalently to the proteins, are lost during the electrophoresis. ^{73}As that remained in the proteins after SDS PAGE indicated that this metalloid must be firmly bound, either covalently or as arsenosugar or as one of the chemically active methylated compounds of trivalent arsenic.

For further characterization of the arsenic-containing proteins present in the homogenates of lung epithelial cells, two-dimensional gel electrophoresis was applied. This method provides stronger denaturing conditions (8 M urea) than SDS-PAGE and it may eliminate all arsenic not very firmly bound to proteins.

The autoradiograms of the lung homogenates from A549 and CCL cells are shown below.

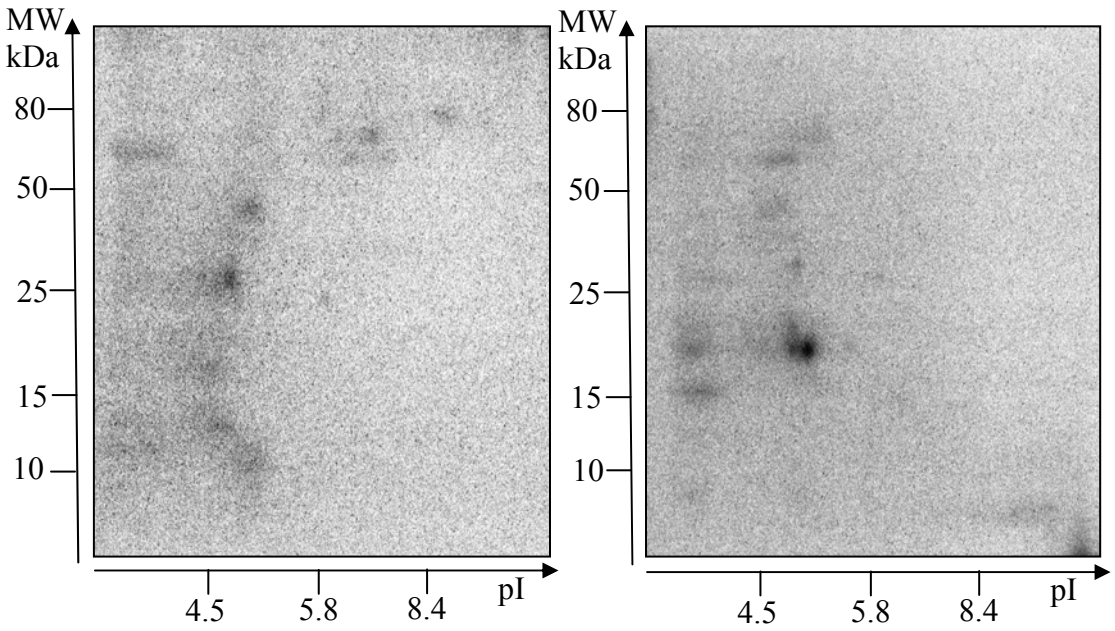

Figure 6-12 Autoradiograms of ^{73}As-labeled proteins in the homogenate of the lung epithelial cells from human (left) and rat (right) after IEF/SDS-PAGE

In the human lung epithelial cells after 2D electrophoresis several spots with molecular masses between 80 and 10 kDa, and pI values between 3 - 10 were found. In the human lung epithelial cells the following spots could be detected. Their pI value are listed in brackets: one at 75 kDa (8.2), three at 60 - 55 kDa (7.5 – 7.9), one at 37 kDa (5.0 -5.2), one at 25 kDa (4.9), one diffuse at 16 kDa (4.8), two at 12 – 10 (4.8 – 5.0). In the rat lung epithelial cells spots

could be seen at 80 kDa (5.4 – 5.5), at 75 kDa (4.9 -5.0), at 50 kDa (4.8 - 4.9), at 25 kDa (4.9 – 5.0) and at 20 kDa (4.9 – 5.0).

6.4.3 Studies on the ^{73}As-labeled metabolites in the cytosols and the culture medium of the cultured cells by means of HPLC and scintillation detector

For studies on the arsenic metabolites, the human lung epithelial cells A549, rat epithelial cells CCL, and human tracheal fibroblasts CRL7422, cultured in FBS-free medium, were labeled in vitro with 410 kBq ^{73}As per flask (after platting $1.5 \times 10^4 – 4 \times 10^4$cells / cm^2 in the flask). The tracer was applied to the medium 24 hours after platting and the cells were incubated for another 24 hours. After that the culture medium was removed and the cell monolayer was rinsed with sterile phosphate-buffer saline (PBS) and than harvested by scraping. The cells were homogenized in 20 mM Tris-HCl pH 7.5 by sonification. The homogenates were separated into pellet and cytosolic fraction by centrifugation for one hour at 100000 × g. The cytosolic proteins and the proteins present in the culture medium were separated by HPLC using a Superdex 75PC column (see section 2.2.4.7) according to differences in their native molecular masses. 33 fractions were collected. The activity of the tracer present in those fractions was measured by a scintillation detector. The following chromatograms show the UV-spectra obtained at 254 and 280 nm and the activity of the tracer in the chromatographically separated samples of the cell cytosols and the culture medium.

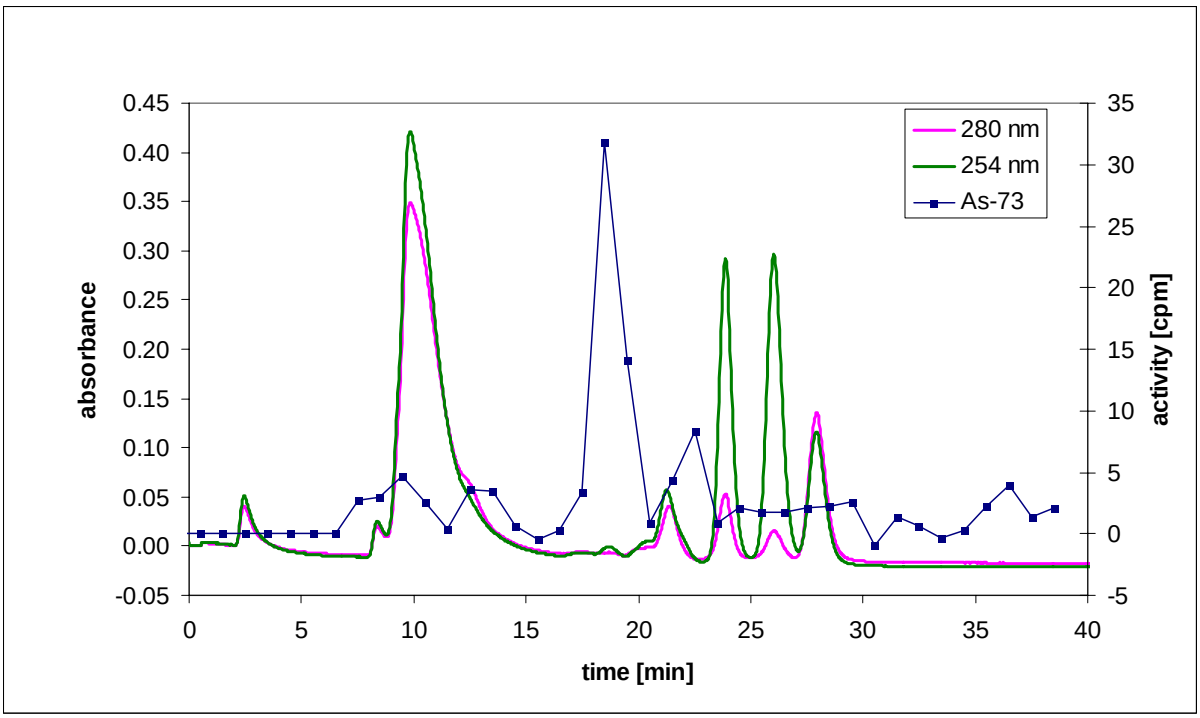

Figure 6-13 UV-profile at 254 and 280 nm and ^{73}As activity in the fractions of the chromatographically separated proteins of the cytosol from human lung epithelial cells

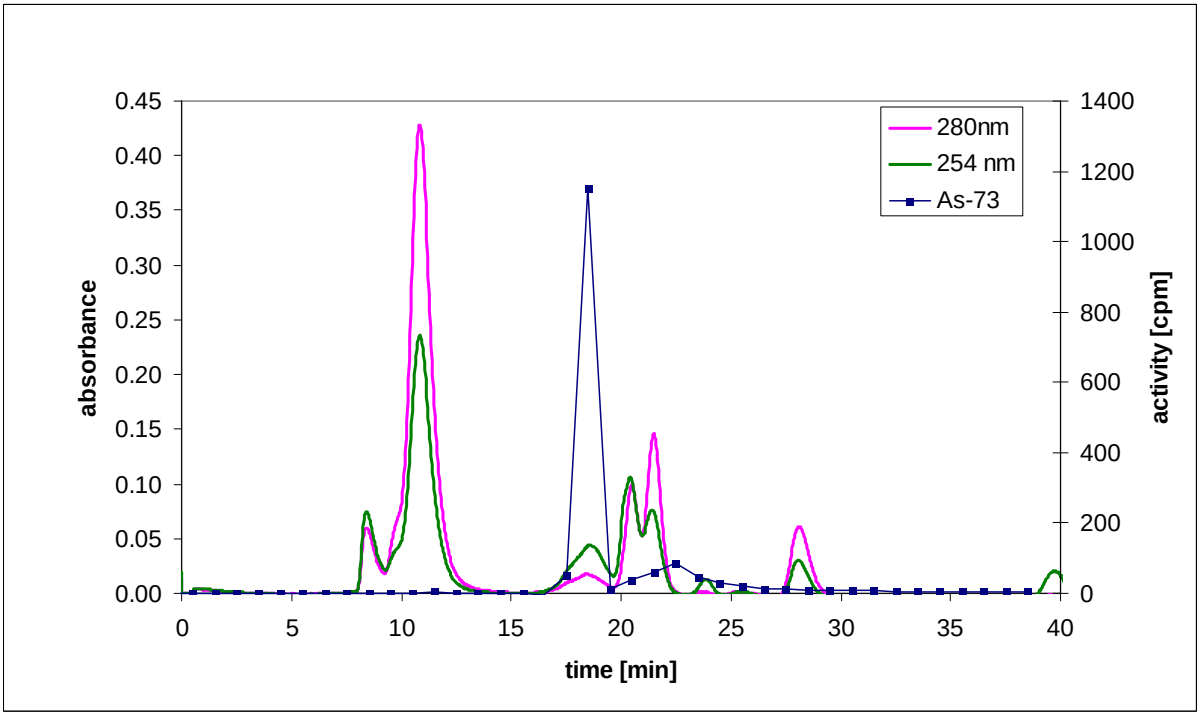

Figure 6-14 UV-profile at 254 and 280 nm and ^{73}As activity in the fractions of the chromatographically separated proteins of the culture medium from human lung epithelial cells

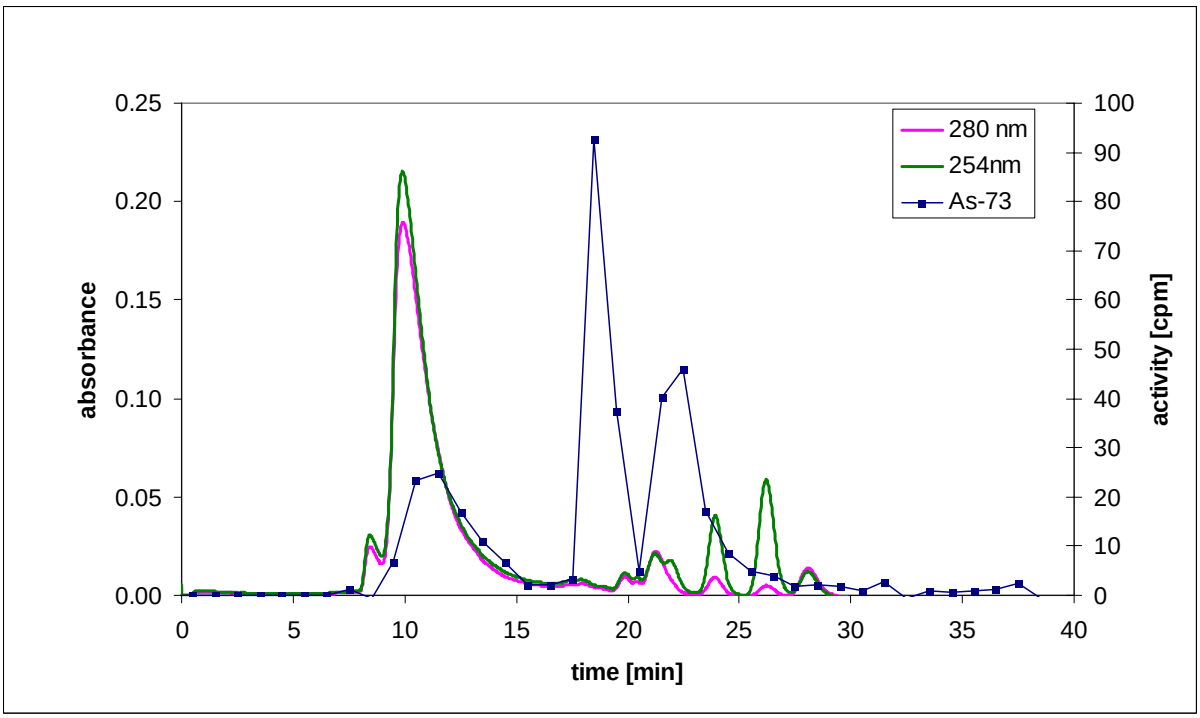

Figure 6-15 UV-profiles at 254 and 280 nm and ^{73}As activity in the fractions of the chromatographically separated proteins of the cytosol from rat lung epithelial cells

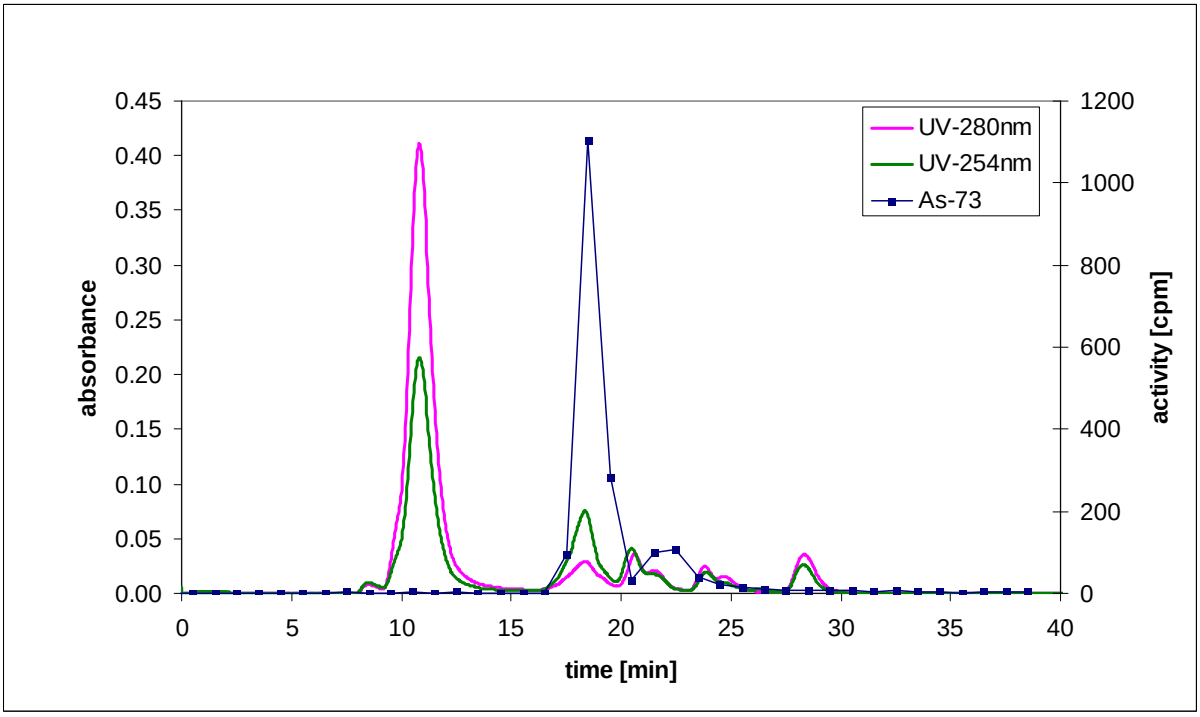

Figure 6-16 UV-profiles at 254 and 280 nm and ^{73}As activity in the fractions of the chromatographically separated proteins of the culture medium from rat lung epithelial cells

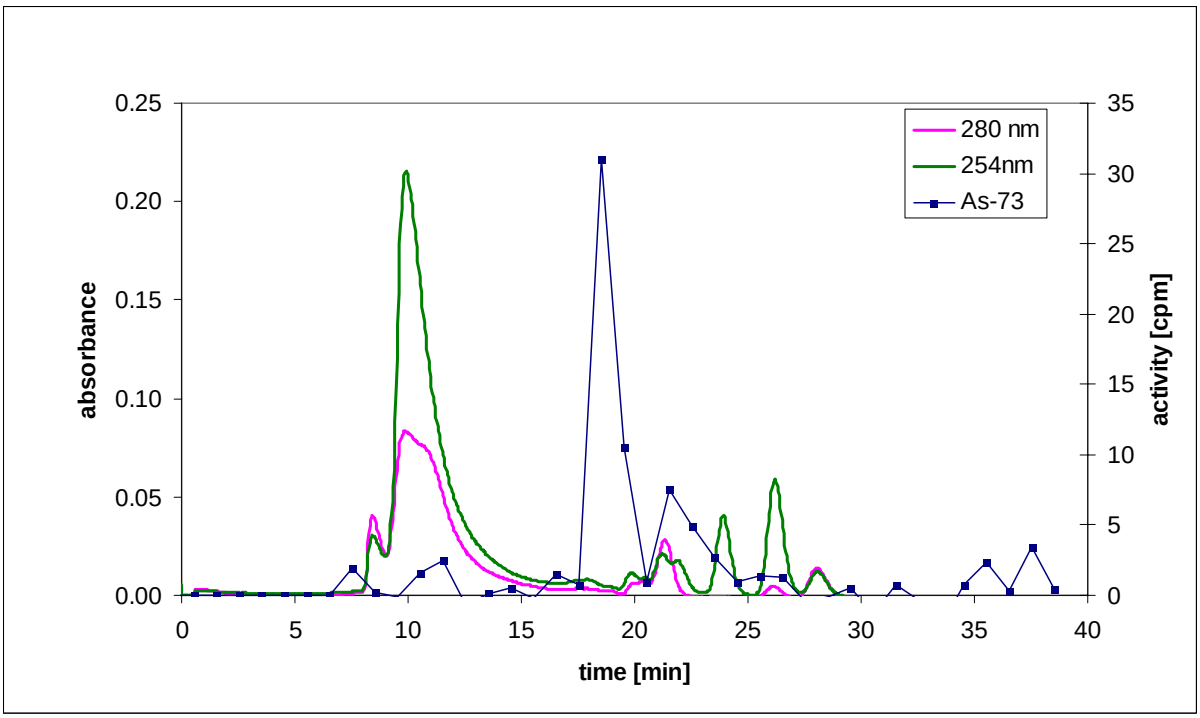

Figure 6-17 UV-profiles at 254 and 280 nm and ^{73}As activity in the fractions of the chromatographically separated proteins of the cytosol from human tracheal fibroblasts

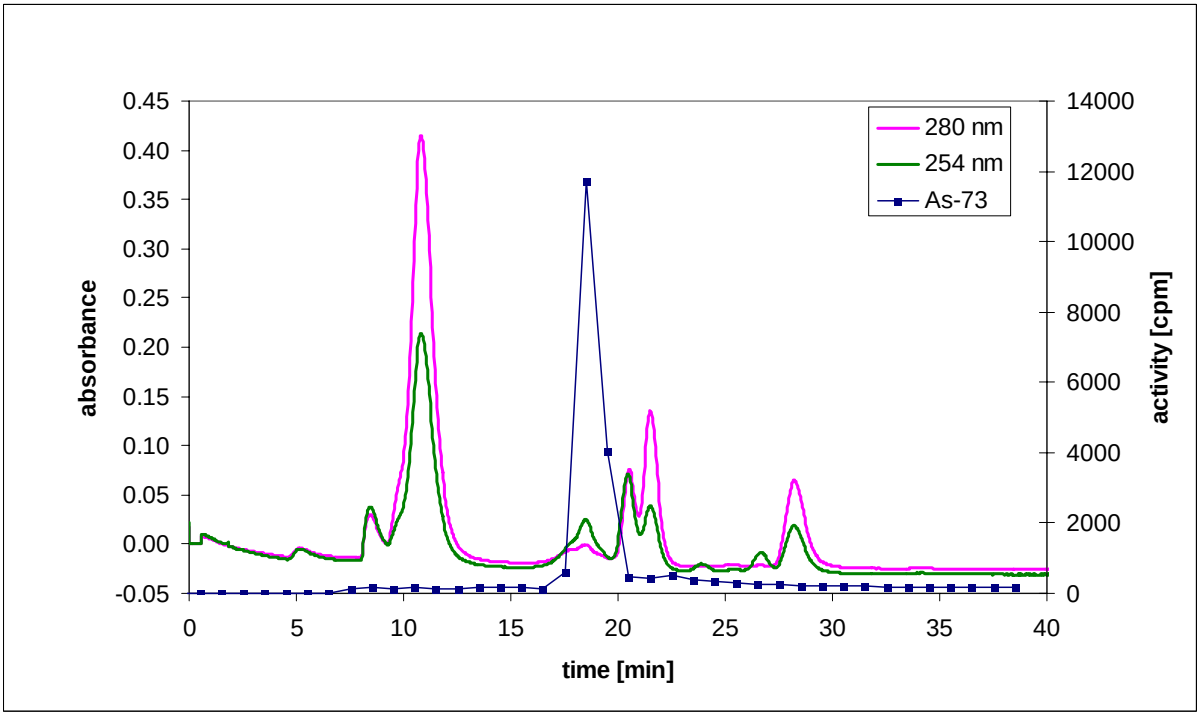

Figure 6-18 UV-profiles at 254 and 280 nm and ^{73}As activity in the fractions of the chromatographically separated proteins of the culture medium from human tracheal fibroblasts

In the fractions of the cytosol from human lung epithelial cells eluted at 9.5 min, 13.5 min, 18.5 min and 22,5 min an increased activity was observed, while in the culture medium from those cells a signal with a very high intensity was found only at 18.5 min. In the cytosol of rat lung epithelial cells three peaks with increasing arsenic activity in the separated fractions were noted. The first peak appeared between 9.5 and 11.5 min, the second at 18.5 min, and the third between 21.5 and 22.5 min. The second one contained most of the tracer activity present in this sample. In the culture medium only one peak at 18.5 min was found. In the cytosol and medium of tracheal fibroblasts the activity of ^{73}As eluted at 18.5 min. In the cytosol an additional peak at 21.5 min was observed.

These results showed that in all fractions a peak appeared at a retention time of 18.5 min. It had the strongest activity in the culture medium of all cells types. It may therefore stem from the same arsenic metabolite (protein). In the cytosols of the cells the activity of the tracer was high in the fraction that contained proteins with higher molecular masses (collected at the beginning of the separation). The proteins present in the separated fractions could not be investigated further by SDS-PAGE or 2D electrophoresis, because of the low tracer activity. The native molecular mass of the proteins present in the separated ^{73}As-containing fraction were determined by calibration of the column used in the experiment.

6.4.3.1 Calibration of the column

The base of the calibration of the Superdex 75PC column is a logarithmic correlation between retention time and molecular mass (shape) of the molecules. This allows the determination of the native molecular mass of proteins. Non-globular shape and/or possible interaction with the size exclusion chromatography (SEC) pose severe limitations with regard to the use of SEC for this estimation. Furthermore, the separation is strongly dependent on the pH value and ionic strength of the eluate [135].

The applied substances are listed in Table 6-1. The calibration curve is shown in Figure 6-19. The exclusion limit was at 9.7 min for dextrane blue and the permeation limit at 19.2 min for vitamin B12. The linear separation range was located between 10 and 19 min. The regression line for this linear correlation was:

$$\textbf{log molecular mass [g/mol] = - 0.1475 · retention time [min] + 3.3806}$$

Table 6-1 Molecular masses and retention time of the substances used in the calibration of the Superdex 75PC column

Substance	Molecular mass [kDa]	Retention time [min]	Log MM
Vit B12	1.3	19.18	0.1139
Aprotinin	6.5	17.51	0.8129
Ribonuclease A	13.7	14.68	1.1367
Chymotrypsinogen A	25	13.94	1.3979
Ovalbumin	43	11.56	1.6334
Albumin	67	10.72	1.8260
Dextrane blue	2000	9.71	3.3010

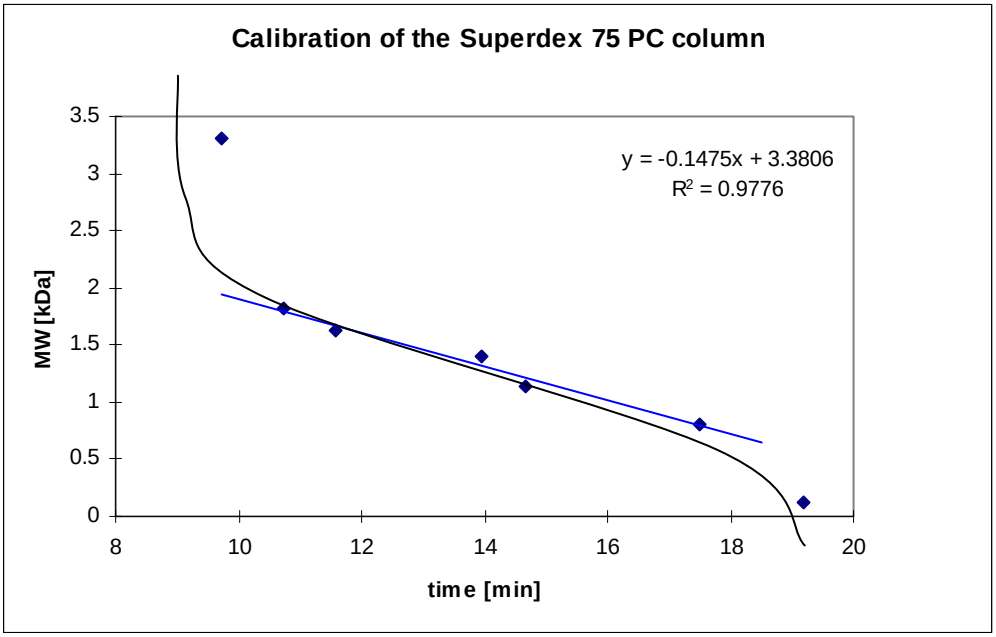

Figure 6-19 Correlation between retention time and molecular mass of several substances (see Table 6-1) separated on a Superdex 75PC column

6.4.3.2 Determination of the molecular masses of separated biomolecules containing ^{73}As

After calibration of the Superdex 75PC column and determination of the retention time for each peak- fraction containing ^{73}As, the molecular masses of the protein or proteins present in the fraction was estimated. The retention time and the calculated molecular mass are listed in the Table 6-2.

Table 6-2 Retention time and molecular mass of the ^{73}As-containing peak after separation on the Superdex 75PC column

Peak	Retention time [min]	log MM	Molecular mass [kDa]
1	9.5	1.97935	95
2	10.5	1.83185	67.9
3	11.5	1.68435	48.3
4	18.5	0.65185	< 6

The molecular mass of the proteins from the fractions eluted at 18.5 min or later could not be established, because it was already outside the leaner range. It could only be estimated that the molecular mass of those proteins was smaller than 6 kDa. For the other three fractions molecular masses of 95 kDa, 67.9 kDa, and 48.3 kDa were calculated. The experiments carried out so far have indicated that arsenic-binding proteins exist in the cytosolic fractions of human and rat endothelial cells and human tracheal fibroblasts. Further studies are needed to investigate more closely the biological function of arsenic and especially its role in those cells.

6.5 Identification of the arsenic-binding proteins in the rat thymus by MALDI-MS

The proteins present in the homogenates of the rat tissues were separated by 2D electrophoresis. The gels were stained using a method of Rabilloud [115], dried and the arsenic-binding proteins were then identified autoradiographically via the ^{75}As tracer. Only in the autoradiogram (Figure 6-20) obtained from thymus homogenate various spots were detected, with molecular masses between 80 kDa and 10 kDa and pI values between 3.5 and 9.5. Two arsenic-containing spots could be localized in the silver-stained gel. The spots with pI values of 7.98 and 9.04 were cut out and after trypsin digestion, their peptides were used for the MALDI-MS analysis.

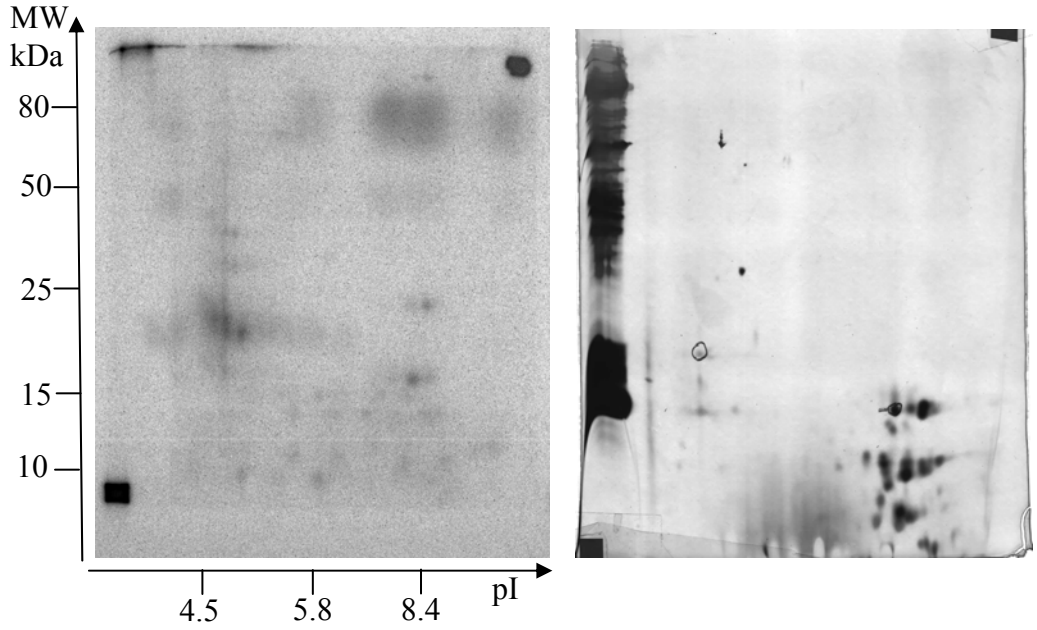

Figure 6-20 Autoradiogram of the ^{73}As-labeled proteins in homogenates of the thymus (left) after IEF/SDS-PAGE and the silver-stained gel (right)

For the quest of the spots the database MSDB 20040106 (1319480 sequences; 419227754 residues) was used. In the case of the first spot, the top score (119) was for Q9QUT6 (NCBI BLAST - www.ncbi.nlm.nih.gov/blast/Blast/cgi), described as beta 1 globin.- Rattus sp. with the molecular mass of 15824 Da and a pI value of 7.98. The search parameters for the spot are shown in the Table 6-3.

Table 6-3 Search parameter used for the protein identification by means of MALDI

Type of search	Peptide mass finger print
Enzyme	Trypsin
Variable modification	Oxidation
Mass values	Monoisotopic
Protein mass	Unrestricted
Peptide mass tolerance	± 300 ppm
Peptide charge state	1+
Max missed cleavages	1
Number of queries	22

The probability based mowse score is shown in the Figure 6-21.

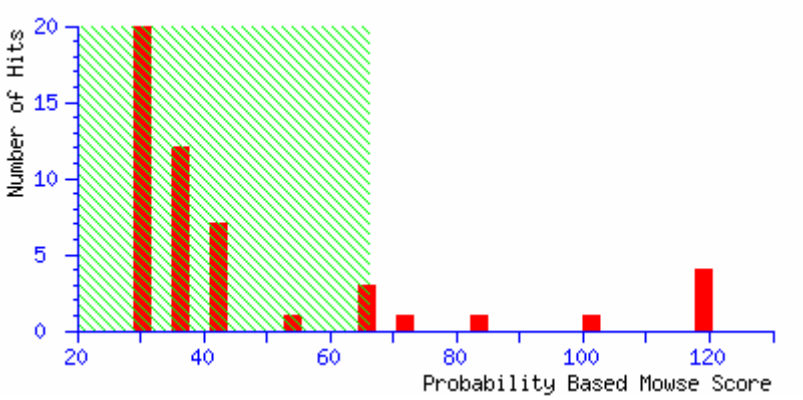

Figure 6-21 The probability-based mowse score; Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 66 are significant ($p<0.05$)

The number of mass values searched was 22 and the number of mass values matched was 10, whereas the sequence coverage achieved 58%. The matched peptides (in red) and appropriate sequences found in the analyzed samples are shown below:

1 VHLTDAEK**AA VNGLWGKVNP DDVGGEALGR LLVVYPWTQR YFDSFGDLSS**
51 **ASAIMGNPK**V KAHGK**KVINA FNDGLK**HLDN LKGTFAHLSE LHCDK**LHVDP**
101 **ENFR**LLGNMI VIVLGHHLGK EFSPCAQAAF QK**VVAGVASA LAHKYH**

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
9 - 30	2195.13	2194.13	2194.11	0.02	1	AAVNGLWGKVNPDDVGGEALGR
18 - 30	1298.73	1297.73	1297.63	0.11	0	VNPDDVGGEALGR
31 - 40	1274.84	1273.84	1273.72	0.12	0	LLVVYPWTQR
41 - 59	2006.91	2005.90	2005.91	-0.01	0	YFDSFGDLSSASAIMGNPK
41 - 59	2022.89	2021.89	2021.90	-0.01	0	YFDSFGDLSSASAIMGNPK
Oxidation (M)						
66 - 76	1218.82	1217.82	1217.68	0.14	1	KVINAFNDGLK
67 - 76	1090.86	1089.85	1089.58	0.27	0	VINAFNDGLK
96 - 104	1126.79	1125.79	1125.56	0.23	0	LHVDPENFR
133 - 144	1122.87	1121.87	1121.66	0.22	0	VVAGVASALAHK
133 - 146	1422.83	1421.82	1421.78	0.05	1	VVAGVASALAHKYH

No match to: 990.00, 1013.88, 1087.90, 1278.83, 1290.82, 1306.81, 1325.82, 1572.76, 1735.86, 2199.12, 2227.13, 2297.16

Table 6-4 Search parameter used for the protein identification by means of MALDI

Type of search	Peptide mass finger print
Enzyme	Trypsin
Variable modification	Oxidation (M), oxidation (HW), propionamide (C)
Mass values	Monoisotopic
Protein mass	Unrestricted
Peptide mass tolerance	± 300 ppm
Peptide charge state	1+
Max missed cleavages	1
Number of queries	23

The search parameters for the second spot found in the 2D gels are shown in the Table 6-4. This spot was identified with a score of 94 as S00840, described as hemoglobin beta chain (minor – rat). The nominal mass was 15982 and the calculated pI value 9.04. The probability based mowes score is shown in the figure below.

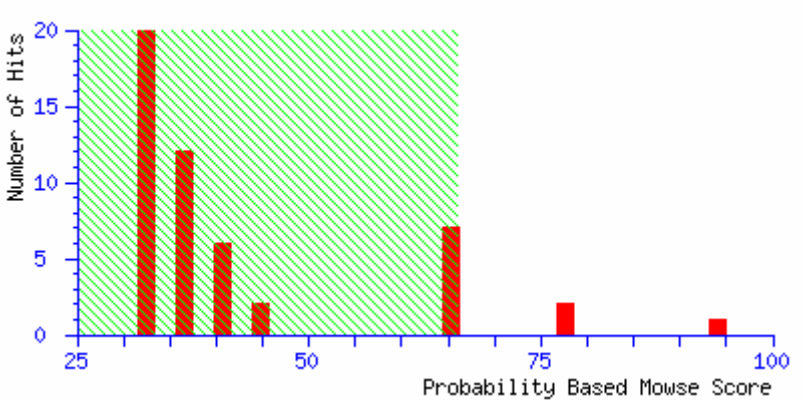

Figure 6-22 The probability based mowse score; Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 66 are significant ($p<0.05$)

In this case the number of mass values searched was 23 and the number of mass values matched was 9, whereas the sequence coverage achieved 55%. The matched peptides (in red) and appropriate sequences found in the analyzed samples are shown below:

1 M**VHLTDAEKA TVSGLWGK**VN PDNVGAEALG RLLVVYPWTQ RYFSKFGDLS
51 SASAIMGNPQ VKAHGK**KVIN AFNDGLKHLD NLKGTFAHLS ELHCDKLHVD**
101 **PENFR**LLGNM IVIVLGHHLG K**EFTPSAQAA FQKVVAGVAS ALAHKYH**

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
2 - 18	1843.74	1842.74	1842.95	-0.21	1	VHLTDAEKATVSGLWGK 2 Oxidation (HW)
67 - 77	1218.59	1217.59	1217.68	-0.09	1	KVINAFNDGLK
68 - 77	1090.60	1089.59	1089.58	0.01	0	VINAFNDGLK
68 - 83	1810.86	1809.86	1809.97	-0.11	1	VINAFNDGLKHLDNLK
78 - 96	2297.17	2296.17	2296.09	0.08	1	HLDNLKGTFAHLSELHCDK 3 Oxidation (HW); Propionamide (C)
84 - 105	2636.40	2635.40	2635.26	0.14	1	GTFAHLSELHCDKLHVDPENFR Propionamide (C)
97 - 105	1126.54	1125.54	1125.56	-0.01	0	LHVDPENFR
122 - 133	1324.53	1323.53	1323.65	-0.12	0	EFTPSAQAAFQK
134 - 147	1422.61	1421.61	1421.78	-0.17	1	VVAGVASALAHKYH

No match to: 959.71, 1072.67, 1087.65, 1109.59, 1234.60, 1242.61, 1471.59, 1735.72, 1859.73, 1865.76, 1938.99, 2279.16, 2319.20, 2566.44

These two arsenic-containing spots found in the gel after 2D electrophoresis were identified by means of MALDI as beta 1 globin and hemoglobin. So far nothing is known about the role of these proteins in arsenic metabolism. Hemoglobin is the iron-containing oxygen-transport

metalloprotein in the red blood cells of mammals. The molecule consists of globin, the apoprotein and four heme groups, with an iron atom in each of them.

6.6 Discussion

Arsenic is a human carcinogen, but there is no substantial evidence of carcinogenicity in other animal species. It has been proposed that the toxicity of arsenic is due to its affinity to the thiol groups of proteins. It has been reported that after administration of arsenic to cells it initially binds to cellular proteins before reduction or methylation can occur [136, 137]. Therefore, the binding of arsenic to cellular proteins is a key determinate in arsenic metabolism [136]. Previous studies showed that arsenic binds to cellular proteins or enzymes in both animal tissues and in vitro cells cultures [137, 138]. Although numerous studies have attempted to isolate arsenic-binding proteins, no specific arsenic-binding proteins have so far been identified in mammalian tissues. Three proteins with molecular masses of 100 kDa, 450 kDa and >2000 kDa were isolated by Bogdan et al. [136], but they were not clearly identified. Metzel et al. detected two-arsenic binding proteins 'tubulin' and 'actin' using arsenical-based affinity chromatography [138]. Chang et al. suggested that two proteins Gal-1 (plays an important role in cell proliferation and apoptosis) and TPX-II (an antioxidant enzyme) act as arsenic-binding proteins in the CHO (Chinese hamster ovary) cells [139].

The comparison of the patterns of the retention of ^{73}As in the whole body and tissues in the two groups of rats fed either a selenium-deficient or selenium-sufficient diet after a single tracer administration showed that the administration of the element was strongly dependent on the selenium status. The distribution pattern of the orally administered arsenic in the rat tissues differs from that after i.p. injection. In the tissues obtained from animals after oral administration clearly less ^{73}As was found. Once absorbed arsenic is bound to haemoglobin, leucocytes, and plasma proteins, it is cleared from the intravascular space within 24 hours and distributed in most tissues [70, 71]. Arsenic compounds, well absorbed by the gastro-intestinal tract or by intra-peritoneal injection, were rapidly distributed to organs or tissues rich in proteins containing sulfhydryl groups, and accumulated mainly in liver, kidney, spleen, lung and adrenal gland [73]. These tissues were mainly labeled. The differences in the ^{73}As distribution in the organs after i.p. and oral administration can be explained by the difference in the arsenic validity in the organism after different administration. In the case of i.p. arsenic administration the element is directly absorbed by the organs in the abdominal cavity and by the vessels system, with blood reached fast other organs. The pathway of arsenic from the gastro-intestinal tract to blood is more complicated. It depends on many factors (stomach and

intestinal linings act as a barrier for many large or charged compounds). It therefore takes longer to reach the tissues. 48 hours after i.p. administration most of the arsenic was found in the pancreas, diaphragm, thymus, blood and spleen, whereas after oral arsenic administration the blood had the highest tracer content.

Remarkable differences in ^{73}As distribution were observed between selenium-deficient and selenium-sufficient animals, with a much higher percentage of the tracer dose being retained in the tissues of the selenium-deficient animals. An indirect, but significant function of selenium is to protect the cell from the oxidative stress and free radical formation that occurs during exercise. Selenium can be considered as the "rate-limiting" substrate in the glutathione (GSH) system. Without selenium the peroxidase enzyme cannot be formed and consequently the antioxidant protection by the Gpx system is decreased. Reduced NADPH, along with glutathione reductase as a catalyst, converts oxidized glutathione back to the reduced form. With increasing free radical peroxidation the ratio of reduced glutathione to oxidized glutathione decreases, hence the ratio is often used as a marker of radical formation within the body [19]. The endogenous increase in the reduced glutathione antioxidant defense system with chronic training likely acts as a protective mechanism to help to prevent oxidative stress within the body. Furthermore, it has been established that in selenium deficiency the Gpx activity decreases. This may lead to the increase in the ratio of the ratio of reduced glutathione to oxidized glutathione. This means that in selenium deficiency more free sulfhydryl residues are formed. This fact may explain the higher retention of arsenic in the selenium-deficient tissues.

In these studies, SDS-PAGE profiles showed several proteins binding radioactive ^{73}As in all tissues. In this way some arsenic-containing proteins could be distinguished. They had molecular masses in the range between 10 kDa and >250 kDa. It was also possible to identify two arsenic-binding proteins in the thymus - beta 1 globin and hemoglobin. So far nothing is known about the role of these proteins in arsenic metabolism. Hemoglobin is the iron-containing oxygen-transport metalloprotein in the red blood cells of mammals. The molecule consists of globin, the apoprotein and four heme groups, with an iron atom in each of them. The high binding affinity of the globin and hemoglobin for arsenicals is probably due to large number of cysteine residues. The rat hemoglobin is a tetramers, consisting of two α chains and two β chains. The rat hemoglobin has three cysteines in each α chain (Cys104, Cys111, and Cys13) and two cysteines in each β chain (Cys93 and Cys125). However, arsenic exist in several forms in the living organism, unclear is also which arsenic species is bind to these proteins.

The differences found in the distribution of the arsenic-binding proteins in the tissues and among their cellular compartments indicate that the arsenic compounds may be involved in different intracellular processes. However nothing is known so far about further arsenic-binding proteins found after SDS-PAGE, about their structure, and function and how arsenic is incorporated into them. Further studies in this research area are therefore needed.