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COPPER RESORPTION IN ISOLATED RAT HEPATOCYTES

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Abstract

Copper plays an important role both in physiology and pathology. In ionic form Cu^{++} is believed to be toxic. Our aim is to investigate the Cu-uptake by isolated rat hepatocytes in an in-vitro experiment. Hepatocytes are cultured on foils to form cellular monolayers, which are exposed to $CuSO_4$ solution. The trace elements P, S, Cl, K, Ca, Fe, Cu, Zn and Br are determined by PIXE, sweeping the proton microbeam in two dimensions across selected regions of the cell cultures. The concentration averages over positions covering the interior of hepatocytes or the intercellular gaps are formed and the behaviour of the various trace elements is studied as a function of the copper solution exposure time. In most cases cell nuclei are identified and evaluated separately.

KUPFER-AUFNAHME IN ISOLIERTEN HEPATOZYTEN VON RATTEN

Zusammenfassung

Kupfer spielt eine wichtige Rolle in der Physiologie und der Pathologie. In ionischer Form wird Cu^{++} für toxisch gehalten. Unser Ziel ist es, die Cu-Aufnahme in isolierten Rattenhepatozyten in einem Laborexperiment zu untersuchen. Dazu werden Leberzellen auf Folien in einer einlagigen Schicht gezüchtet und einer Kupfersulfat-Lösung ausgesetzt. Die Spurenelemente P, S, Cl, K, Ca, Fe, Cu, Zn und Br werden mit Protonen-induzierter Röntgenstrahlung (PIXE) bestimmt, wobei der anregende Protonen-Mikrostrahl zeilenförmig über ausgewählte Bereiche der Zellkulturen bewegt wird. Die Konzentrationen werden über alle Messpositionen gemittelt, die das Innere der Leberzellen oder die Zellzwischenräume überdecken. Untersucht wird das Verhalten der verschiedenen Spurenelemente in Abhängigkeit der Zeit, während der die Probe der Kupfersulfat-Lösung ausgesetzt wird. In den meisten Fällen können die Zellkerne identifiziert und gesondert ausgewertet werden.

1. Introduction

Copper plays a still unclear part in liver cirrhoses. In primary biliary cirrhosis a high content of copper is observed in hepatocytes which line fibrosed portal tracts [1,2,3]. Similar findings exist for cirrhoses with other ethiologies (alcohol toxic, hemochromatosis) [3,4], which also show copper accumulations in the region of active inflammation adjacent to the fibrotically degenerated portal area. In Morbus Wilson the copper concentration in the liver is elevated, but in contrast with the former diseases, the copper is homogeneously distributed in the latter case [5]. In order to enlighten the situation we tried to simulate the influence of copper on hepatocytes by an in-vitro experiment: Hepatocytes are cultured as a mono-layer on thin foils and exposed to $CuSO_4$ solution. By the metabolic function of the hepatocytes the copper should be taken up into the cells and excreted again. This might be observed by the analysis method of micro-PIXE (proton induced X-ray emission), which is sensitive enough to detect copper concentrations in the µg/g range with a spatial resolution smaller than the 15 µm size of the hepatocytes.

In a completely different approach [6,7,8] similar studies on the resorption of Cu from the plasma have been performed using the radioactive tracer ^{64}Cu. They revealed a saturation kinetics and a temperature dependency for the Cu-uptake process. These observations are explained with a facilitated transport by a special carrier protein, which also transports Zn, in combination with diffusional uptake.

2. Sample Preparation

After anesthesia with pentobarbital (60 mg/kg body weight), hepatocytes are isolated from the livers of male Wistar rats with 300 g weight according to a collagenase method of Berry and Friend [9]. Formvar® foils of 30 µg/cm^2 thickness are stretched over the 5 mm diameter holes of the aluminium support frames and coated with collagen to enable the hepatocytes to attach. The isolated hepatocytes are suspended in a modified Leibovitz L15 medium containing 12.5 mmol/l HEPES, 12.5 mmol/l $NaHCO_3$, 8.3 mmol/l D(+)glucose, 5 mmol/l L(+)lactate, 1%(v/v) antibiotic/antimycotic solution, and 10%(v/v) fetal bovine serum. The hepatocytes grow as a monolayer on the Formvar® foil with a density of 900 cells/mm^2 in an humidified incubator under standard conditions (atmosphere 95% air and 5% CO_2 at 37° C). For the first 6 h of cultivation 1 µmol/l dexamethason and 25 µmol/l insulin are added. After 6 h the medium is changed, containing now additionally (1 µmol/l each) testosterone, 3,3',5-triiodo-L-thyronine, (-)-epinephrine, epidermal growth factor, glucagon, and β-estradiol, but no insulin. Shortly before the treatment with copper solution, the viability of the hepatocytes is tested by staining with trypan blue (0.34 mmol/l, 2 min in L-15), which marks unviable hepatocytes.

To study the effects of copper uptake, the cell cultures are incubated with a solution of 30 µmol/l $CuSO_4$ and 30 µmol/l L-histidine in a buffered L-15 medium (pH 7.4 at 37° C) free from hormones under continuous stirring for time periods ranging from < 1 sec to 30 min. Immediately after removing the cells from the solution, the remaining medium is sucked off and the hepatocytes are washed twice with Cu-free medium at 4° C to prevent a further Cu-

uptake. After drying of the samples, groups of 5 - 6 vital hepatocytes within a 70 x 60 μm^2 area are selected for irradiation with the proton microbeam. As the cells are completely carbonized by the irradiation, a microphoto using Nomarski interference contrast is taken before the irradiation to allow a correlation with the collected data. One sample is prepared in the same manner, but without $CuSO_4$ incubation, and serves as a control group.

3. Beam Exposure

Nine cell cultures have been irradiated in the Karlsruhe ion microprobe setup [10] with a 3 MeV proton beam of ≈ 2.5 µm diameter at currents between 150 and 300 pA. The two-dimensional scans with 32 x 32 points cover areas of 70 x 60 μm^2. The fast scanning speed of 2 mm/sec reduces the local heating of the samples. The meandrous pattern [11] is repeated many thousand times to accumulate sufficient X-ray counts in each pixel. Of special interest are positions where neighbouring cells come into contact and form bile canaliculi, as here metabolic products are excreted from the cells. The proton induced X-rays are registered with a Si(Li) detector (190 eV resolution, 80 mm^2 active area, 5 mm thickness, 22 mm distance from target). Fig. 1 gives the X-ray spectrum summed over all scanned points of the sample exposed for 20 min to the $CuSO_4$ solution.

The matrix thickness distribution is derived [12] from the protons which are elastically backscattered from C, N, and O and recorded in an annular silicon surface barrier detector (100 mm^2 active area, 100 µm depletion depth, 47 mm distance from target) mounted in the backward direction. The summed backscattering

spectrum of the 20 min sample is presented in fig. 2. For an absolute concentration calibration we use a 577 $\mu g/cm^2$ thick plastic foil (pure $(CH_2)_n$) on which a 57.3 $\mu g/cm^2$ Ni-layer is evaporated.

4. Data Reduction

Maps of the trace elements P, S, Cl, K, Ca, Fe, Cu, Zn, and Br are produced by integration over the K_α peaks of the trace element in question and subtracting background by a suitable choice of background gates below and above the K_α peak energy. This relatively simple procedure is performed for each of the 32 x 32 pixels instead of the more precise peak fitting which would cost considerably more in computer time. For each pixel the organic matrix mass is determined from the local proton backscattering count rate falling into the gate indicated in fig. 2. To get the local concentration we divide the X-ray intensity by the matrix mass and normalize it [12]. With the average element composition of liver [13] the thickness dependent matrix correction is taken into account [12,14] for each position. Fig. 3 shows a set of trace element and matrix thickness maps of a sample exposed for 2 min to the $CuSO_4$ solution, together with a microphoto taken with Nomarski interference contrast before irradiation.

Regions of arbitrary shape covering irradiated positions with the same histological structure (hepatocytes, bile canaliculi) are defined with the aid of the microphoto. For the sample exposed for 2 min to the $CuSO_4$ solution, these regions are indicated in fig. 4 by different letters. Within each region the trace element concentrations are averaged. The scattering of the individual

values around the average value reveals the standard deviation. In most cases cell nuclei are identified and evaluated separately. From the set of samples with different $CuSO_4$ exposure periods the time behaviour of various trace elements is derived.

5. Results

The concentration values (in µg/g dry matter) of the measurable trace elements in all 9 samples under investigation are collected in the tables 1 to 4. Some of the results are already published elsewhere [15,16]. The control group and the sample with exposure time < 1 sec show normal trace element concentrations [17,18]. Fig. 5 gives the time dependent Cu-concentration in the hepatocytes and in the bile canaliculi. Obviously the Cu-uptake is not yet finished after 30 min. The cell nuclei show the same behaviour, which is given in fig. 6. In contrast, the Cu-concentration in the bile canaliculi declines after 20 min. Similar findings for the Cu-uptake are observed by a completely different method using the radioactive tracer ^{64}Cu [19].

During the incubation of the samples, the pH value tends to increase. To keep it at 7.4, varying amounts of HCl are added during the $CuSO_4$ exposure, which explain the extremely high and strongly fluctuating chlorine concentrations. The low energy tail of the Cl K_α line in the X-ray spectrum adds to the sulphur peak. Unfortunately the background subtraction technique does not completely compensate this contribution of the tail at excessive Cl-concentrations. Therefore the observed correlation of sulphur with chlorine is a data evaluation artefact. Perhaps the HCl-solution is contaminated with potassium, as the K-concentrations go

up and down in parallel with the Cl-levels. Unviable cells in the scanned region exhibit Ca-levels which are significantly increased (up to ≃ 3000 µg/g) compared with those of viable hepatocytes (≃ 1500 µg/g). Fig. 7 displays the Zn concentration levels, which exhibit no significant time behaviour. Similarly the concentration levels of P, Fe and Br remain unchanged and do not seem to be involved in the copper transport mechanism.

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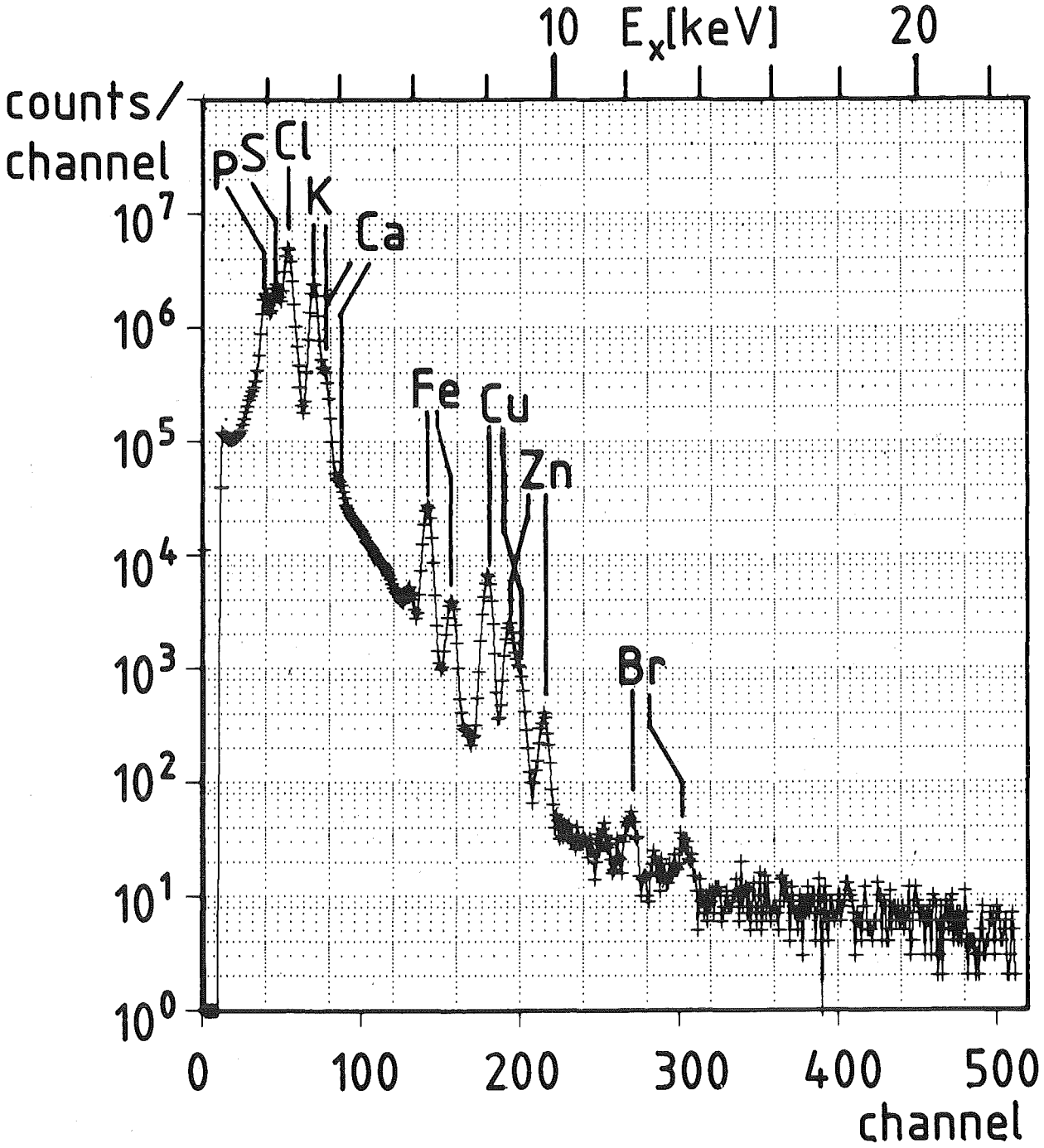

Fig. 1. PIXE spectrum of the 20 min sample summed over all scanned points.

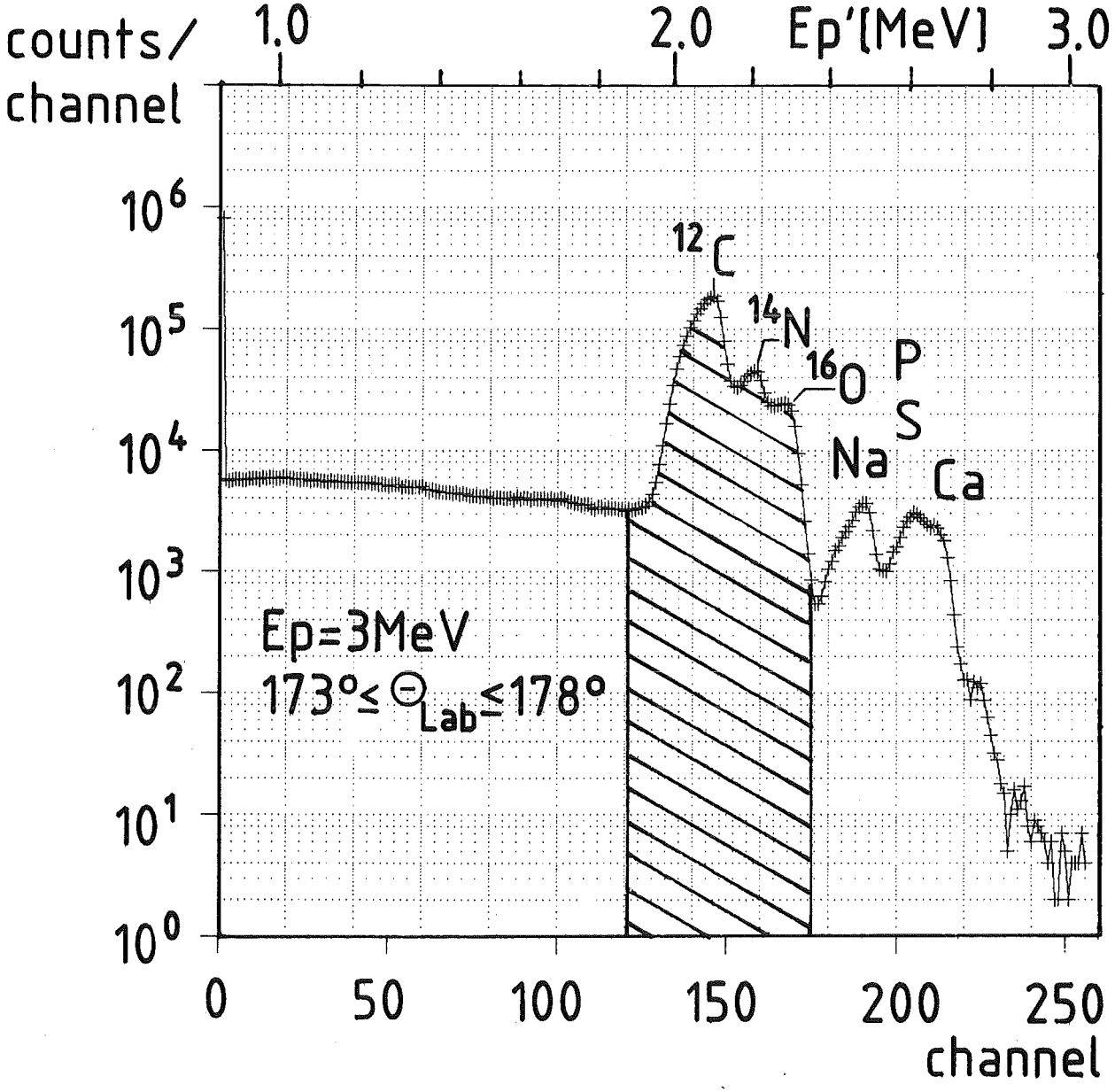

Fig. 2. Backscattering spectrum of the 20 min sample summed over all scanned points.

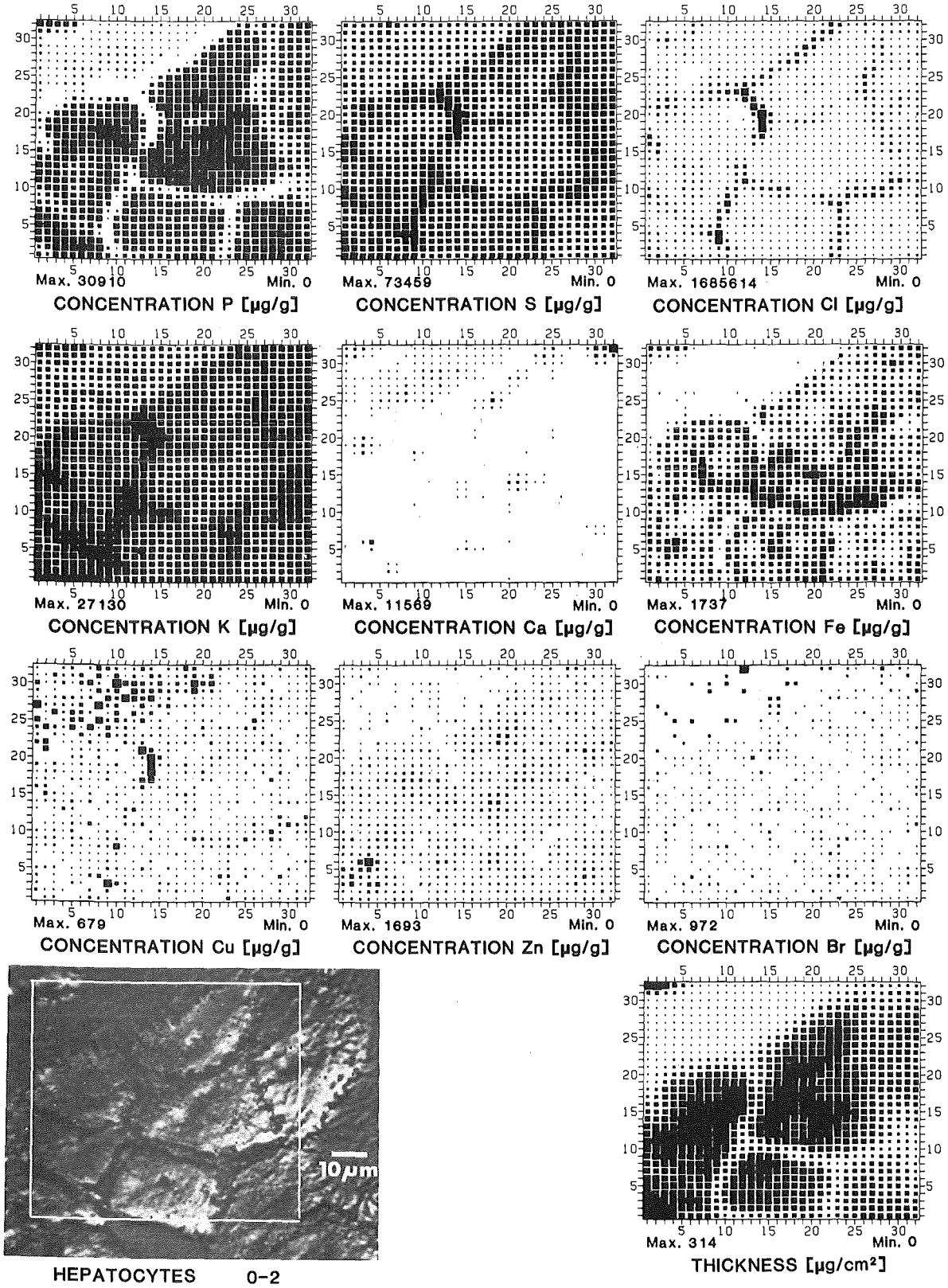

Fig. 3. Trace element and matrix thickness distributions in the 70 x 60 µm² scanned area of the 2 min sample. Concentration values are normalized relative to the carbon hydride matrix mass.

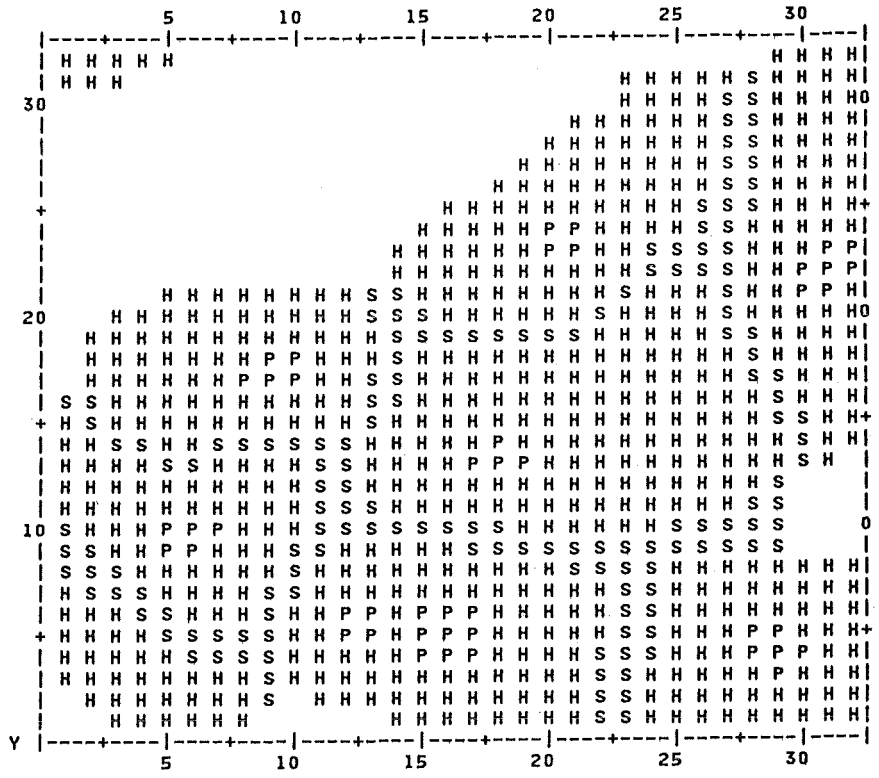

Fig. 4. Distribution of positions with the same histological structure as used for the averaging of the concentration values on the sample which is exposed for 2 min to the $CuSO_4$ solution. H = hepatocytes, S = bile canaliculi, P = cell nuclei.

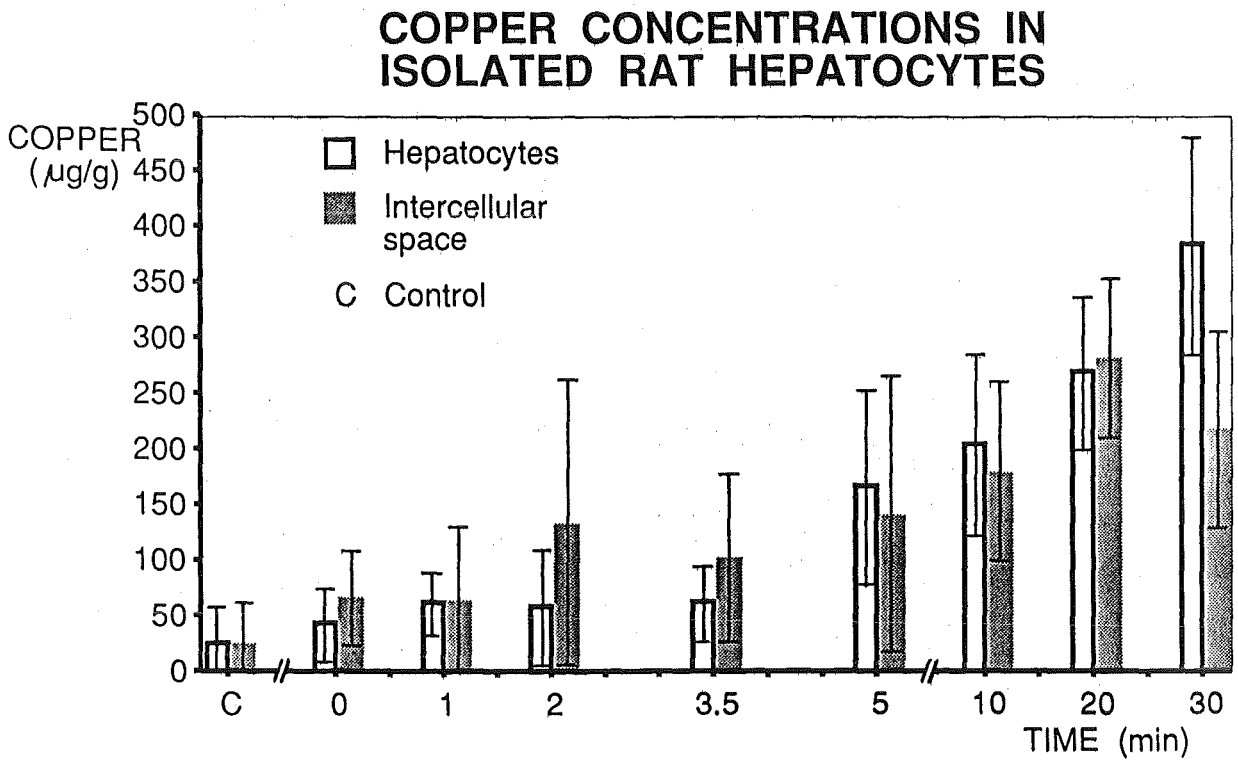

Fig. 5. Time dependent concentrations of copper in isolated rat hepatocytes and in the bile canaliculi.

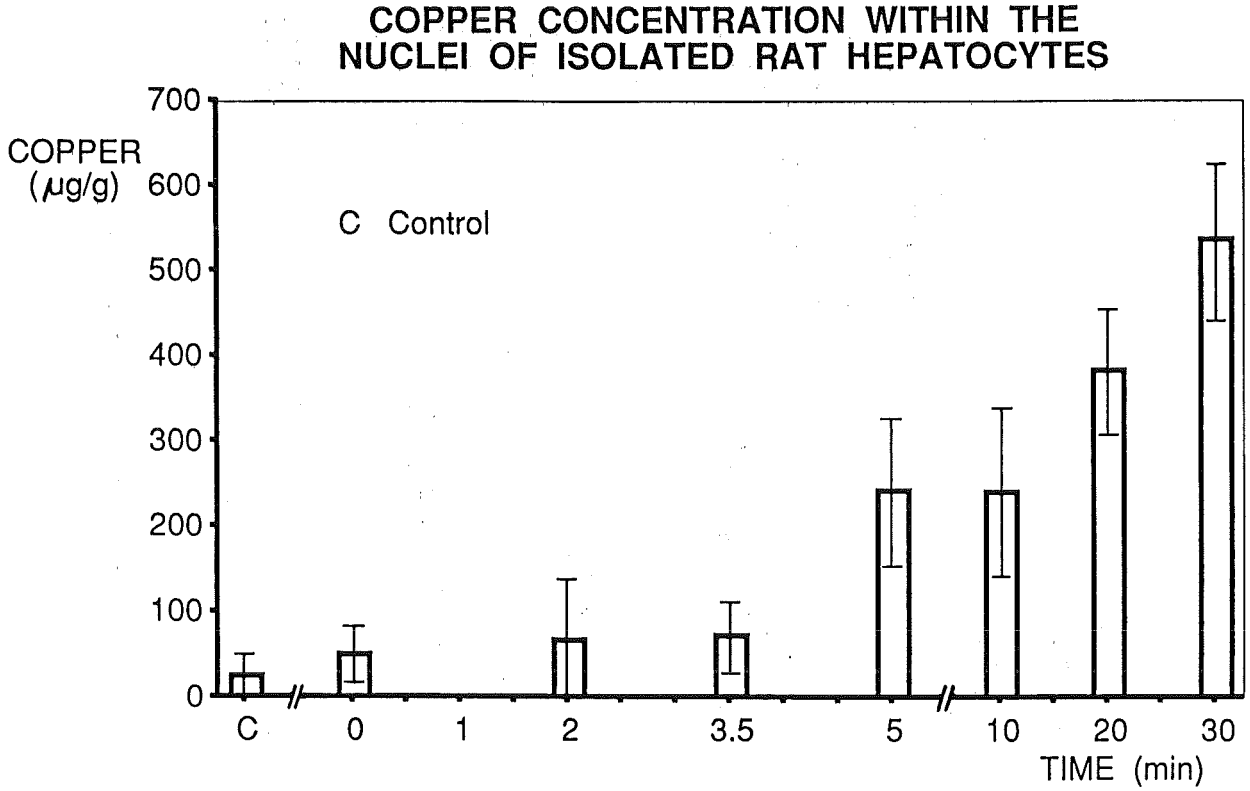

Fig. 6. Time dependent concentrations of copper within the nuclei of isolated rat hepatocytes.

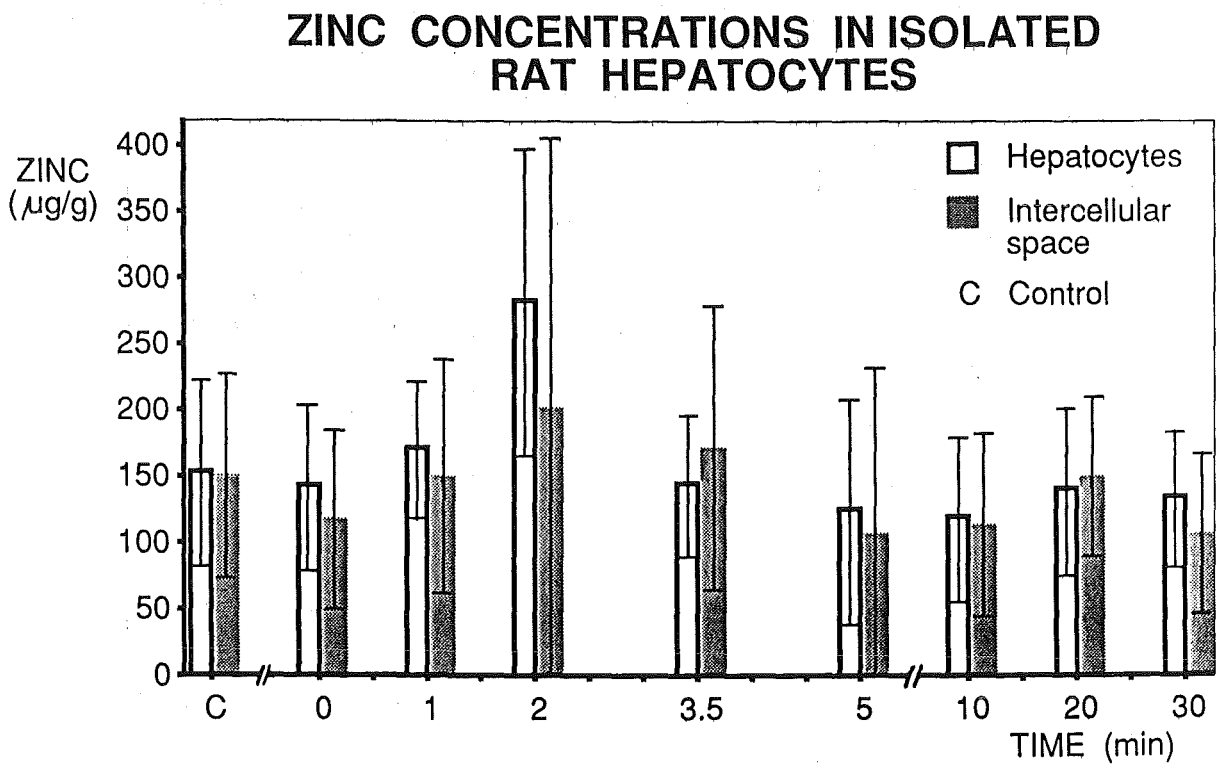

Fig. 7. Time dependent concentrations of zinc in isolated rat hepatocytes and in the bile canaliculi.

Table 1. Trace element concentrations relative to carbon hydride in the liver cells [in µg/g].

element	control	exposure time 0 min	1 min [1])	2 min	3.5 min
P	16086 ±888	10632 ±2117	17458 ±1188	22076 ±3349	15629 ±874
S	16843 ±727	21484 ±1692	19419 ±1114	42414 ±4752	13246 ±645
Cl	70830 ±7064	91174 ±25902	67648 ±4917	210179 ±75416	27032 ±2074
K	11831 ±1395	10460 ±802	10334 ±567	19061 ±1743	7501 ±449
Ca	621 ±135			3490 ±1319	
Fe	376 ±145	399 ±170	488 ±161	832 ±268	435 ±172
Cu	27.6 ±33.3	48.1 ±34.2	66.4 ±30.7	63.1 ±54.0	66.9 ±33.5
Zn	157 ±69.6	147 ±61.7	175 ±52.6	287 ±116	149 ±53.6
Br	11.7 ±41.8	18.3 ±43.8	11.2 ±30.7	55.1 ±68.5	10.4 ±32.5
thickness [µg/cm²]	386 ±75.0	321 ±97.6	488 ±102	218 ±55.7	278 ±62.0

[1]) Concentration values of this sample include the cell nuclei.

Table 1.(cont.) Trace element concentrations relative to carbon hydride in the liver cells [in µg/g].

element	exposure time 5 min	10 min	20 min	30 min
P	17666 ±1287	14929 ±1105	13607 ±3097	14544 ±2470
S	34510 ±5519	25370 ±2554	11689 ±1155	14668 ±2459
Cl	215814 ±62521	131799 ±22611	22086 ±1412	45381 ±6842
K	15218 ±2539	8820 ±954	10245 ±749	8063 ±840
Ca	737 ±635	564 ±198	1702 ±301	1633 ±1098
Fe	251 ±162	353 ±151	584 ±190	537 ±253
Cu	171 ±88.6	209 ±82.0	274 ±71.3	389 ±99.2
Zn	130 ±85.2	124 ±62.4	145 ±63.3	139 ±52.0
Br	35.5 ±89.1	26.9 ±58.5	8.3 ±28.2	8.0 ±25.6
thick-ness [µg/cm^2]	270 ±76.5	345 ±62.5	358 ±143	406 ±184

Table 2. Trace element concentrations relative to carbon hydride in the intercellular gaps [in µg/g].

element	control	exposure time 0 min	1 min	2 min	3.5 min
P	15383 ±1007	6505 ±3148	14065 ±2112	11064 ±9063	13543 ±1992
S	17969 ±1024	21495 ±2070	17076 ±2160	52250 ±6223	13781 ±1552
Cl	81311 ±9743	210983 ±80104	60852 ±9105	562765 ±259598	30639 ±9407
K	17474 ±2390	9895 ±1024	9544 ±1086	21522 ±2012	7678 ±551
Ca	872 ±202			2915 ±1150	
Fe	396 ±152	257 ±145	505 ±170	707 ±327	756 ±488
Cu	27.1 ±37.5	68.6 ±43.5	66.2 ±68.5	135 ±132	105 ±75.3
Zn	153 ±76.2	120 ±67.8	152 ±88.3	204 ±201	174 ±108
Br	13.0 ±53.8	23.4 ±46.9	8.5 ±66.0	72.4 ±94.9	0.45 ±70.1
thickness [µg/cm²]	288 ±78.5	243 ±64.9	147 ±75.2	171 ±53.8	156 ±89.7

Table 2.(cont.) Trace element concentrations relative to carbon hydride in the intercellular gaps [in µg/g].

element	exposure time 5 min	10 min	20 min	30 min
P	19909 ±1933	14885 ±793	14630 ±2799	9637 ±1538
S	49685 ±9258	26656 ±2514	11197 ±824	9680 ±1172
Cl	456148 ±180621	150695 ±25712	22966 ±1513	34552 ±2708
K	20688 ±9006	9221 ±840	10758 ±666	6492 ±492
Ca	59.3 ±745	566 ±171	1775 ±175	1051 ±110
Fe	133 ±223	323 ±143	671 ±183	452 ±175
Cu	143 ±125	181 ±82.0	284 ±72.0	220 ±88.9
Zn	110 ±124	116 ±70.0	152 ±60.3	109 ±61.1
Br	27.6 ±134	30.1 ±93.7	5.5 ±28.4	6.9 ±57.9
thickness [µg/cm²]	164 ±46.4	232 ±51.8	268 ±88.0	134 ±21.6

Table 3. Trace element concentrations relative to carbon hydride in the cell nuclei [in µg/g].

element	control	exposure time 0 min	1 min [1])	2 min	3.5 min
P	17330 ±877	13248 ±1189		22334 ±4168	17233 ±1063
S	17073 ±582	21921 ±1578		43869 ±5673	13349 ±400
Cl	71508 ±9219	104608 ±17083		271310 ±95178	27602 ±1886
K	12349 ±1408	11091 ±569		19236 ±1591	7672 ±452
Ca	635 ±107			3575 ±856	
Fe	304 ±172	176 ±108		507 ±372	351 ±169
Cu	29.9 ±28.3	55.2 ±34.3		71.5 ±72.8	77.1 ±40.8
Zn	249 ±70.0	274 ±70.4		416 ±143	233 ±70.7
Br	0.4 ±33.4	20.6 ±30.3		48.3 ±71.8	15.2 ±33.7
thickness [µg/cm²]	386 ±65.9	365 ±48.4		228 ±42.9	285 ±50.0

[1]) Cell nuclei are not resolved in this sample.

Table 3.(cont.) Trace element concentrations relative to carbon hydride in the cell nuclei [in µg/g].

element	exposure time 5 min	10 min	20 min	30 min
P	18653 ±1178	15147 ±731	16022 ±975	16473 ±1196
S	32525 ±4675	25736 ±2137	12200 ±350	16601 ±1095
Cl	199181 ±51296	129665 ±16765	24093 ±1182	50562 ±2088
K	14634 ±1131	9197 ±901	11105 ±556	9015 ±519
Ca	512 ±311	661 ±247	1712 ±103	1342 ±297
Fe	189 ±136	302 ±160	426 ±135	485 ±210
Cu	247 ±87.0	245 ±98.7	388 ±72.8	541 ±91.8
Zn	243 ±84.4	215 ±52.3	211 ±68.4	202 ±29.6
Br	24.3 ±67.6	37.5 ±58.2	7.2 ±24.3	10,8 ±19.7
thickness [µg/cm²]	348 ±38,4	363 ±65.9	353 ±80.9	559 ±128

Table 4. Trace element concentrations relative to carbon hydride in unviable hepatocytes [in µg/g].

element	control	exposure time 0 min	1 min	2 min [1])	3.5 min
P	14836 +2163	8952 +2430	16272 +2632		16363 +980
S	18242 +2142	26212 +2798	18550 +1070		12412 +660
Cl	81869 +11951	133139 +34870	57510 +2609		27545 +7742
K	15741 +4278	11801 +1083	9555 +349		7235 +302
Ca	1302 +485	1962 +1592	2638 +1396		1412 +805
Fe	330 +180	119 +116	623 +211		494 +113
Cu	44.4 +54.6	107 +54.9	72.3 +46.9		48.8 +32.8
Zn	132 +85.7	61.9 +47.3	145 +59.3		99.0 +37.0
Br	7.0 +68.8	16.0 +41.7	8.1 +49.5		8.7 +27.8
thick-ness [µg/cm²]	294 ±188	287 ±77.2	453 ±223		304 ±46.7

[1]) Only viable hepatocytes in the scanned area.

Table 4.(cont.) Trace element concentrations relative to carbon hydride in unviable hepatocytes [in µg/g].

element	exposure time 5 min [1])	10 min	20 min	30 min
P		14803 +986	10978 +3831	17585 +4362
S		28202 +2563	11214 +2105	12187 +2198
Cl		160909 +24745	21095 +2823	42555 +4109
K		9546 +832	10020 +1366	7715 +839
Ca		1174 +372	2953 +1301	9902 +3873
Fe		281 +144	337 +170	162 +168
Cu		179 +76.8	242 +83.6	229 +94.2
Zn		106 +69.9	87.6 +66.6	46.5 +47.8
Br		30.0 +84.7	11.7 +49.4	11.5 +38.3
thickness [µg/cm²]		241 ±55.8	208 ±107	229 ±136

[1]) Only viable hepatocytes in the scanned area.