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Determination of zinc-65, copper-64 and sulphur-35 labelled rat hepatoma tissue culture metallothioneins by high-performance liquid chromatography with on-line radioactivity detection

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ABSTRACT

Molecular size exclusion (MSE), reversed-phase (RP), and anion-exchange (AE) high-performance liquid chromatography (HPLC) techniques were employed in combination with on-line radioactivity detection, in a study on the kinetic behaviour of ^{65}Zn-, ^{64}Cu- and [^{35}S]cystein-labelled metallothionein (MT) in rat hepatoma tissue culture (HTC) cells. MSE-HPLC of [^{35}S]cysteine-labelled HTC cell cytosol resulted in co-eluting MT-I and MT-II isoforms (t_R 19.80 min; V_c/V_o: 1.85). AE-HPLC of ^{65}Zn-treated HTC cell cytosol yielded separated ^{65}Zn MT-I (t_R 11.5 min; I = 64 mM) and ^{65}Zn MT-II (t_R 14.5 min; I = 104 mM). RP-HPLC of ^{64}Cu-treated HTC cytosol resulted in separated ^{64}Cu MT-I (t_R 26.4 min) and ^{64}Cu MT-II (t_R 23.4 min). Determination of the amino acid composition, apparent molecular mass and cysteine content of HTC MT-I and MT-II isoforms showed the characteristics of class I metallothioneins. The rate of dissociation of Zn^{2+} from Zn-MT could be determined from the losses of ^{65}Zn from MT during a single AE-HPLC run, showing a Zn-MT dissociation half-life of 0.66 h. RP-HPLC showed a delay in incorporation of newly accumulated ^{64}Cu into MT, possibly owing to the appearance of reduced glutathione as an intracellular copper-transfer compound. Application of compartmental analysis in [^{35}S]-cysteine accumulation experiments permitted the determination of the actual rate of MT degradation; when 200 μM of Zn were applied, the MT degradation half-life was 2.0 ± 0.8 h. These results indicate the potential of combined HPLC techniques and application of radionuclides in studies on the synthesis and degradation of MT and metal-MT complexes.

INTRODUCTION

Since the first isolation of a cadmium-binding protein from equine renal cortex by Margoshes and Vallee [1], metallothionein (MT) has been isolated from a wide range of human and animal tissues, such as human liver, fetal, neonatal and adult rat liver, rat brain, and bovine hippocampus [2–5]. Non-mammalian MT-like proteins have been isolated from fish, marine invertebrates, yeast, microorganisms and plants (for review see ref. [6]).

The chemical and physiological properties of MT isolated from these diverse sources, in particular mammalian MTs, are very similar, showing an extensive sequence homology, particularly with regard to the location on the 20–22 metal-binding cysteinyl residues along the polypeptide chain [6]. MTs may be classified as a group of sulphydryl-rich cytosolic proteins with a low molecular mass (M_r: 6000–6800), which can bind a variety of heavy metals, and induced by stress, physiological and nutritional factors [7–10]. Al-

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most all mammalian MTs consist of at least two major isoforms: MT-I and MT-II. These proteins are similar with respect to sulphydryl content and metal-binding capacities [11]. Because of the conservation of the metal-binding potential throughout evolutional development, MT is thought to play a fundamental role in intracellular zinc and copper homeostasis [12], and also in the detoxification of heavy metals, such as cadmium and mercury [13].

Current methods for the quantification of MT in biological materials are based either on the direct determination of the protein moiety or on the indirect determination by metal and SH content of MT (for review see ref. 14). However, although the metal-saturation assays, radioimmunoassays, and electrochemical procedures are useful for the quantification of total MT, they fail to distinguish between MT isoforms. The growing number of reports on the application of high-performance liquid chromatography (HPLC) techniques (gel permeation [15], anion-exchange [16], or reversed-phase [17–19]) may indicate the general interest in these MT isoforms. Moreover, the combined use of HPLC and atomic absorption spectroscopy (AAS) [18] and/or inductively coupled plasma (ICP) techniques [20] permitted the detection of metals associated with MT.

This paper addresses the various HPLC techniques in a study on MT in rat hepatoma tissue culture (HTC) cells [21,22], and describes the combined use of HPLC and on-line radioactivity detection [23,24]. The HTC MT isoforms I and II were isolated, purified, and further characterized by amino acid analysis. Experiments were carried out bij radiolabelling with [^{35}S]cysteine, ^{64}Cu or ^{65}Zn, under MT-inducing conditions. Simultaneous quantitative analysis of MT and radiotracers indicated both the resolving power of the HPLC techniques applied, and the specific advantages of the use of radioactive isotopes; the latter were shown by quantitative analysis of M^{n+}-MT complexes and by the determination of the actual rate of MT degradation.

EXPERIMENTAL

Radionuclides

^{64}Cu was prepared by irradiating 3.3 mg of copper wire (99.99%; Ventron, Karlsruhe, Germany) in the IRI nuclear reactor for 48 h (thermal neutron flux density $1.6 \cdot 10^{17}$ m^{-2} s^{-1}). After irradiation, the copper wire was dissolved in 25 μl of concentrated HNO_3, and diluted in 3 ml of 50 m*M* sodium acetate buffer (pH 5.6). The final copper concentration was 17.3 m*M*, with a specific activity of 5.2 GBq/mol.

^{65}Zn (as $ZnCl_2$ in 0.1 *M* HCl; 0.37 GBq/mol Zn) was diluted in a 50 m*M* sodium acetate buffer (pH 5.6) to a stock solution with a specific activity of 1.8 MBq/mol.

^{109}Cd (as $CdCl_2$ in 0.1 M HCl; high specific activity 0.4 TBq/mol Cd) was diluted in a sodium acetate buffer (pH 5.6), to a final concentration of 0.1 m*M* Cd^{2+} (specific activity 0.8 GBq/mol Cd).

^{65}Zn, ^{109}Cd, L-[^{35}S]cysteine (43.7 TBq/mmol) and $^{35}SO_4^{2-}$ (7.9 TBq/mmol) were obtained from Amersham Int. (Buckinghamshire, UK).

Chemicals

Chemicals were obtained from the following sources: fetal calf serum, Ham's F-10 medium, glutamine, and penicillin/streptomycin from Flow Labs. (McLean, VA, USA); rabbit MT-I and MT-II, Sigma (St. Louis, MO, USA); Tris and acetonitrile (HPLC grade), Janssen Chimica (Beerse, Belgium); hydrochloric acid, J.T. Baker (Philipsburg, NJ, USA). All buffer chemicals and solvents were purchased as ultrapure or HPLC grade.

Cell cultures

Rat HTC cells (Morris hepatoma 7288C) were obtained from Flow Labs. Cell cultures were inoculated in collagen-coated disposable culture flasks (75 cm^2) for 24 h in 15 ml of growth medium, in a concentration of $1 \cdot 10^5$ cells/ml. The growth medium, containing Ham's F-10 [25], was supplemented with 12% fetal calf serum, 0.2 m*M* glutamine, and penicillin (100 IU/ml)/ streptomycin (0.1 μg/ml). Cells were grown at 37°C in a

humid atmosphere of 5% (v/v) CO_2 in air, until confluency was reached (*ca.* $7.5 \cdot 10^6$ cells/flask).

Cell incubation

HTC cell cultures were incubated for 24 h in growth medium supplemented with the following metal concentrations: (1) for molecular size exclusion experiments, 0.2 m*M* cysteine (spiked with 11.7 MBq L-[^{35}S]cysteine/ml), plus 200 μM Zn; (2) for anion-exchange experiments, 200 μM Zn (spiked with 1.8 MBq ^{65}Zn/mol Zn); (3) for reversed-phase experiments, 200 μM Cu (spiked with 6.5 MBq ^{64}Cu/mol Cu). For MT isolation and amino acid analysis of HTC MT isoforms, cell cultures were incubated for 24 h in a medium containing 1.0 μM Cd^{2+} or 200 μM Zn^{2+}, respectively. After 24 h, cells were scraped off and washed three times with cold phosphate-buffered saline (PBS). Cell suspensions were frozen in liquid nitrogen and thawed three times in 5 vol. 25 m*M* Tris–acetate buffer (pH 7.4), supplemented with 5 m*M* β-mercaptoethanol and aprotinin (1 μg/ml). After ultracentrifugation at 105 000 *g* for 90 min at 4°C, the soluble fraction (cell supernatant) was separated from the particulate fraction (cell membranes and organelles). Aliquots of cell supernatant were directly analysed for MT content and AAS determinations, and further used in HPLC.

Determination of metals

Total zinc and copper concentrations in cell cytosol were analysed by AAS (Perkin-Elmer Model 2380), using an air–acetylene flame and a graphite furnace. Cell cytosol samples were digested in a 1:1 (v/v) mixture of 65% HNO_3 (Suprapur, Merck, Darmstadt, Germany) and 30% hydrogen peroxide at 90°C for 24 h, and finally diluted into a 2% HNO_3 solution. Zinc and copper absorptions were measured at 213.7 nm and 324.6 nm, respectively, with a hollow-cathode lamp and a slit-width of 0.7 nm. Amounts of protein were determined according to the method of Lowry *et al.* [26].

Determination of metallothionein

MT concentrations (containing Zn, Cu and/or Cd) were determined by the Cd/ammonium tetrathiomolybdate assay according to Klein *et al.* [27], and the Cd/Chelex-100 assay, following Bartsch *et al.* [28]. Briefly, MT containing HTC cell cytosol fractions were incubated with a radiolabelled ^{109}Cd solution (3.3 MBq ^{109}Cd/mg Cd; total concentration, 0.1 m*M* Cd^{2+}) for 10 min at room temperature. Excess of ^{109}Cd was removed by Chelex-100, and ^{109}Cd bound to MT was counted with a NaI γ-detector. The total amounts of MT were determined from the total amount of Cd bound to MT, using a Cd/MT molar ratio of 7 *g* atoms Cd per mole MT [29]. For Cu-incubated HTC cell cytosol, the amount of Cu bound to MT was calculated from the difference in the results of the Cd/Chelex assay (which allows determination of Zn-MT and Cd-MT only) and the Cd/tetrathiomolybdate assay. All Cd-MT saturation assays were calibrated with standard amounts of rabbit MT-I.

Radiolabelling of standard MT isoforms

Rabbit MT-I and MT-II samples (0.15 μM MT in Tris–HCl buffer, pH 7.4) were incubated with 0.1 m*M* Cd^{2+} (spiked with 3.7 MBq $^{109}Cd^{2+}$/ml) for 24 h. After that time, excess of Cd^{2+} was removed via Chelex-100 treatment, followed by centrifugation (10 000 *g* for 5 min at 4°C). The ^{109}Cd-radiolabelled MT samples were used for MT position reference in all three HPLC experiments, and for MT recoveries and UV signal responses.

UV detection

Protein UV absorbances from HTC cytosol samples were monitored at 220 nm (anion-exchange HPLC) and 214 nm (reversed-phase HPLC), and the MT peak areas were determined by integration. The relationships between the peak-area absorbance units (AU) and the MT concentrations were determined using calibrated rabbit MT-I and MT-II samples, in amounts from 0.10 to 10 μg of MT.

Recovery of radionuclides

Samples of ^{109}Cd-labelled rabbit MT-I and MT-II were injected into the various columns

used. ^{109}Cd counting in eluate fractions was performed by liquid scintillation counting (LSC). The MT recoveries were determined as the ^{109}Cd activity counted in the MT peaks of the eluate divided by the ^{109}Cd activity in the injected volumes. Furthermore, ^{109}Cd assays [27,28] were carried out on the injected and recovered MT fractions. Column recoveries were determined as the total activity recovered divided by the total activity injected.

HPLC equipment and calibration

The HPLC system consisted of an LKB 2150 HPLC pump with a Rheograde 7125 high-pressure injection valve. Elution was performed with an LKB 2152 LC control unit (LKB-Pharmacia, Bromma, Sweden). The radioactivity detector consisted of a Berthold LB 506C LSC monitor and a Bernard 1 Stadler E.100.1 scintillator pump (Betron Scientific, Rotterdam, Netherlands).

Radioactivity was measured by the admixture method. Liquid scintillation fluid Flo-Scint IV (Packard Radiomatic, Groningen, Netherlands) was aspired with the scintillation pump, at a flow-rate of 7.00 ml/min, and mixed continuously with the HPLC eluate. The mixture was passed through a flow-through cell, and the radioactivity was measured between two photomultipliers operating in coincidence to reduce background counts. Data processing, including peak-area measurements, was performed with the LB 506 C HPLC software.

To determine the efficiency of Berthold LSC, calibrated samples of ^{65}Zn, ^{64}Cu and $^{35}SO_4^{2-}$ were injected onto the Berthold LB 506C LSC monitor in 10–100 μl aliquots (100–2000 Bq), under HPLC conditions. The fractional efficiency, E_f, was calculated as:

$$E_f = \frac{[\text{cts}]\,[\text{total flow}]}{A\,[\text{cell volume}]}\,100\% \qquad (1)$$

with [cts] is the total number of counts in the peak area, the total flow is the volume per unit time (ml/min), A is the applied activity (dpm), and the cell volume is in millilitres. The total recoveries of the ^{35}S, ^{65}Zn or ^{64}Cu radionuclides used in the Berthold efficiency experiments were all greater than 96% (data not shown).

HPLC analysis

Molecular size exclusion. ^{35}S-containing HTC cell cytosol was separated on a TSK G2000 SW analytical column (600 mm × 7.5 mm I.D.), (LKB-Pharmacia). The mobile phase was 100 m*M* Tris–HCl plus 100 m*M* NaCl (pH 7.4) and 0.02% sodium azide. All buffers were prepared using Milli-Q-purified, deionized water. Buffers were degassed with helium, and kept under a constant-pressure helium atmosphere during HPLC. The flow-rate was set at 1.00 ml/min.

DEAE anion exchange. ^{65}Zn-containing HTC cytosol was pre-separated on a TSK G3000 SWG preparative column (600 × 21.5 mm I.D.), (LKB-Pharmacia) The mobile phase was 100 m*M* Tris–HCl plus 100 m*M* NaCl (pH 7.4) and 0.02 % sodium azide. The buffer was degassed with helium, and kept under a constant-pressure helium atmosphere during all HPLC runs. The applied flow-rate was 5.00 ml/min.

^{65}Zn-MT fractions from the pre-separation runs were pooled, and desalted on a PD-10 disposable column (Pharmacia, Bromma, Sweden). Column eluates were pooled, freeze-dried, and dissolved in 2.0 ml of Milli-Q water (Waters, Milford, MA, USA). MT-I and MT-II were separated on a DEAE 5PW anion-exchange column (75 × 7.5 mm I.D.) (Waters). All buffers were prepared using Milli-Q-purified, deionized water. The mobile phase buffers were: buffer A, 20 m*M* Tris–HCl (pH 7.0) plus 0.02% sodium azide; buffer B, 250 m*M* Tris–HCl (pH 7.0) plus 0.02% sodium azide. Both buffers were degassed, and kept under a helium atmosphere during HPLC analysis. Buffer A was used from 0 to 5 min, followed by a linear gradient from buffer A to buffer B from 5 to 20 min. The flow-rate was set at 1.00 ml/min.

Reversed-phase HPLC. ^{64}Cu-labelled HTC cell cytosol was directly chromatographed by RP-HPLC using a μBondapak C_{18} Radial Pack column (100 × 8 mm I.D., 10 μm particle size), housed in a Z-module, equipped with a C_{18} precolumn (Waters). The mobile phase buffers were:

buffer A, 25 m*M* Tris–HCl (pH 7.4); buffer B, 60% acetonitrile in buffer A (pH 7.4). The buffers were prepared using Milli-Q-purified deionized water. The buffers were degassed with helium, and kept under a helium atmosphere during HPLC analysis. The gradient programme was: 0–10% B (5 min), 10–25% B (20 min), 25–60% B (2 min), 60% B (3 min), 60–0% B (5 min). The applied flow-rate was 1.25 ml/min.

Compartmental analysis

In all ^{35}S counting, activities were considered to represent [^{35}S]cysteine. If [^{35}S]cysteine in experiments is taken to be present in the bulk solution, in the cellular non-MT associated pools or in the cellular MT pools, any experimental set-up for [^{35}S]cysteine may be regarded as a closed three-compartment system. Accordingly [30,31], all [^{35}S]cysteine time-curves could be fitted by:

$$q_n(t) = A_1 \cdot \exp(-g_1 \cdot t) + A_2 \cdot \exp(-g_2 \cdot t) + A_3 \quad (2)$$

with $q_n(t)$ as the amount of [^{35}S]cysteine in the n^{th} compartment (n = 1, 2, or 3) at time t, A_1, A_2 and A_3 are compartment constants, and g_1, and g_2 are compartment-independent rate constants. Following Shipley and Clark [30], the unidirectional rate constants k_{AB}, k_{BA}, k_{BC} and k_{CB} were derived from the function constants A_1, A_2, A_3 (n = 1, 2, or 3), g_1 and g_2. Note that, in the present paper, the constants g_1 and g_2 were derived by simultaneous consideration of all relevant compartments.

Amino acid analysis

Soluble fractions (cytosol) from Zn-treated HTC cell cultures (exposed to 200 μM Zn^{2+} for 24 h) were chromatographed on a TSK G3000 SWG preparative column. Eluate fractions (containing MT-I and MT-II) were pooled and desalted on a PD-10 disposable column (Pharmacia) and further separated on a DEAE 5PW anion-exchange column. The individual MT-I and MT-II fractions were pooled, lyophilized and stored at −80°C.

Cytosol fractions from Cd-treated HTC cell cultures (exposed to 1 μM Cd^{2+} for 24 h) were pre-separated on a TSK G3000 SWG preparative column, as described above. Fractions of Cd-MTs were desalted, pooled, lyophilized and stored at −80°C. Amino acid analysis was carried out according to Hirs [32], including a performic acid oxidation of the cysteine residues, 6 *M* HCl acid hydrolysis at 166°C for 2 h, and column chromatography of the *o*-phthalaldehyde (OPA) and 9-fluorenylmethoxycarbonyl (FMOC) derivatives (Eurosequence, Groningen, Netherlands).

RESULTS

On-line radioactivity detection: optimization

^{64}Cu, ^{65}Zn and ^{35}S radioactivity measurements were carried out by liquid scintillation counting using Berthold LSC equipment with a flow-through cell. Optimal conditions (sensitivity and resolution) were established by repeated injections of various amounts of $^{64}Cu^{2+}$, $^{65}Zn^{2+}$ and $^{35}SO_4^{2-}$, which were dissolved in the buffers used in RP-HPLC, MSE-HPLC and AE-HPLC, using various flow-through cell volumes and discriminator settings. Based on the optimization procedures, a 1.0-ml Z-1000-4 flow-through cell was used for all RP-HPLC and AE-HPLC experiments, whereas a 2.0-ml Z-2000-4 flow-through cell was applied during MSE-HPLC conditions.

Under the conditions described, the detection efficiency (Z-2000-4 cell) for ^{35}S was determined as 37 ± 1% (n = 15). Detection efficiencies for ^{65}Zn and ^{64}Cu (Z-1000-4 cells) were obtained as 47 ± 4% (n = 20) and 66 ± 4% (n = 20), respectively.

The absolute detection limit (defined as three times the noise level) was calculated as 3 Bq, equivalent to 0.9 pmol of Cu (based on a ^{64}Cu specific activity of 53 MBq/mg Cu), 0.7 pmol of Zn (based on a ^{65}Zn specific activity of 66 MBq/mg Zn), and 8.0 pmol of S (based on a ^{35}S specific activity of 11 MBq/mg S). Calibration curves (using standard solutions of $^{64}Cu^{2+}$, $^{65}Zn^{2+}$, and $^{35}SO_4^{2-}$ in various Tris–HCl buffers) were found to be linear in the range from 10 to 55 000 Bq (r^2 = 0.995, n = 8), corresponding to concentrations 10^{-7} to 10^{-4} *M* concentrations (specific activities as described above).

Determination of standard rabbit MT-I and MT-II

Cd-saturation assay. Instrumental neutron activation analysis showed the absence of Cu in the commercially purchased standard rabbit MT-I and MT-II (data not shown), therefore the Cd/Chelex saturation assay (*cf.* Bartsch *et al.* [28]) was used in quantitative standard-MT calibrations. The results indicate full agreement between Cd-saturation data and weighed-in masses of MT-I and MT-II. The Cd assay gave a mass ratio of 1.0 ± 0.05 (n = 12), independent of the amount of MT (0.1–10 μg) or isoforms, suggesting the absence of any significant mass-affecting impurities in the MT standard materials. A further check on impurities was carried out by UV measurements of MT-I and MT-II after HPLC.

UV detection. Although the Cd-saturation assays indicated the absence of any measurable impurities in the MT masses, UV detection may reveal impurities that exhibit strong specific UV signals. AE-HPLC of rabbit MT-I and MT-II showed only minor impurities detectable at 220 nm, confirming the results obtained by Cd-saturation. Based on the above, UV calibrations could be carried out against injected masses of MT-I and MT-II. UV signals were linearly related to injected MT, for the total range (0.1–10 μg) of MT masses (data not shown); the data indicate a specific UV signal (peak areas) for injected MT of *ca.* 10^6 absorption units per μg MT, under the described HPLC and UV detection conditions (Table I). The data also indicate the absence of Cd effects on MT-specific UV signals.

HPLC retention times and recoveries. For MSE-HPLC (TSK G2000 SW), ^{109}Cd-labelled rabbit standard MT-I and MT-II co-eluted at V_e/V_0 = 1.86 (t_R = 19.85 min, data not shown). In AE-HPLC experiments, MT-I and MT-II eluted at t_R = 11.6 and 14.4 min (ionic strength I = 67 and 100 mM), respectively (Fig. 1). For RP-HPLC, the retention times were 26.0 and 23.5 min for MT-I and MT-II, respectively (Fig. 2).

For recovery tests, rabbit MT-I or MT-II were ^{109}Cd-assayed, after which the ^{109}Cd-MT complexes were injected. After DEAE-AE chromatography of rabbit MT-I, the fractional recovery of ^{109}Cd was 0.91 ± 0.05 in the MT-I peak and *ca.* 0.05 (as impurities) in the MT-II peak (see Fig. 1); minor amounts ^{109}Cd appeared at higher retention times. Similar results were found for MT-II injections (with minor MT-I impurities); consequently, MT recovery during DEAE-AE chromatography was set at 0.96 ± 0.05. Similar RP-HPLC experiments yielded MT recoveries of 0.95 ± 0.08. The MT recovery during desalting procedures (disposable Sephadex PD-10 columns) was checked by ^{109}Cd assay of injected

TABLE I

RELATIONSHIP OF UV ABSORPTION UNITS (AU) AND METALLOTHIONEIN AMOUNTS IN THE VARIOUS HPLC ANALYSIS METHODS

Species	Sample	Anion exchange[a] (AU × 10^5/μg MT)	Reversed phase[b] (AU × 10^5/μg MT)
Native rabbit	MT-I	9.63 ± 0.70	9.84 ± 0.81
	MT-II	8.70 ± 0.66	11.04 ± 1.10
^{109}Cd-labelled rabbit	MT-I	8.96 ± 0.34	9.98 ± 0.35
	MT-II	7.90 ± 0.53	10.17 ± 0.55
HTC ^{64}Cu	MT-I/MT-II	–	10.36 ± 0.92
HTC ^{65}Zn	MT-I/MT-II	7.96 ± 0.42	–

[a] Wavelength 220 nm.
[b] Wavelength 214 nm.

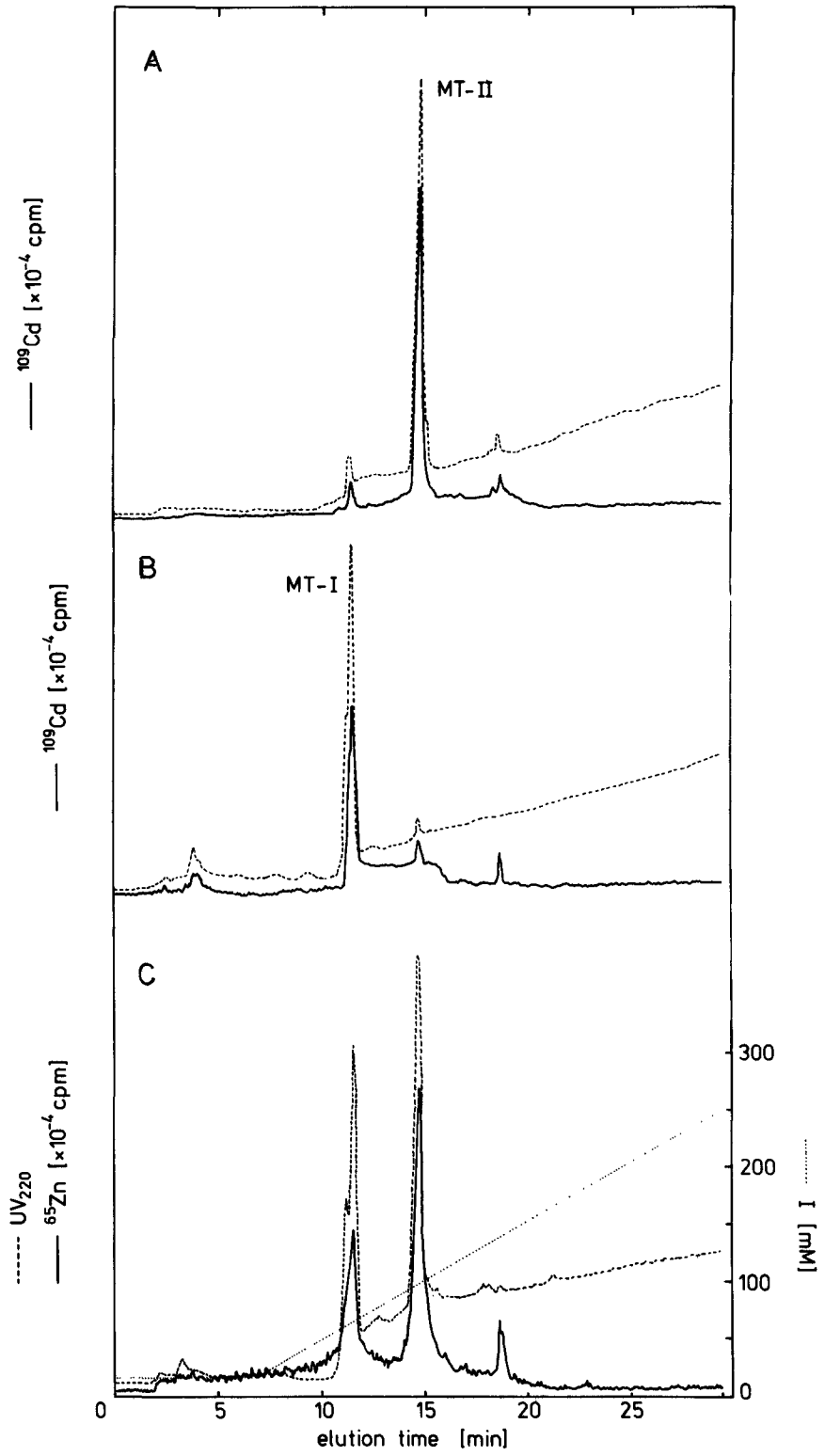

Fig. 1. (A) and (B) Anion-exchange chromatograms of ^{109}Cd-labelled rabbit MT-I and MT-II. Commercially obtained rabbit MT-I and MT-II were radiolabelled by incubation with 0.1 m*M* Cd^{2+} (0.5 MBq ^{109}Cd/mmol Cd), and isolated as described in Experimental. (C) Anion-exchange chromatogram of rat HTC cytosol, pre-separated by MSE HPLC. Cells were incubated in a medium containing 200 μM Zn (spiked with 0.2 MBq ^{65}Zn/mmol Zn) for 24 h. Cell cytosol was obtained as described in Experimental. The retention time of rat HTC MT-I was 11.5 min (I = 64 m*M*) and that of MT-II was 14.5 min (I = 104 m*M*). The UV detection wavelength was 220 nm.

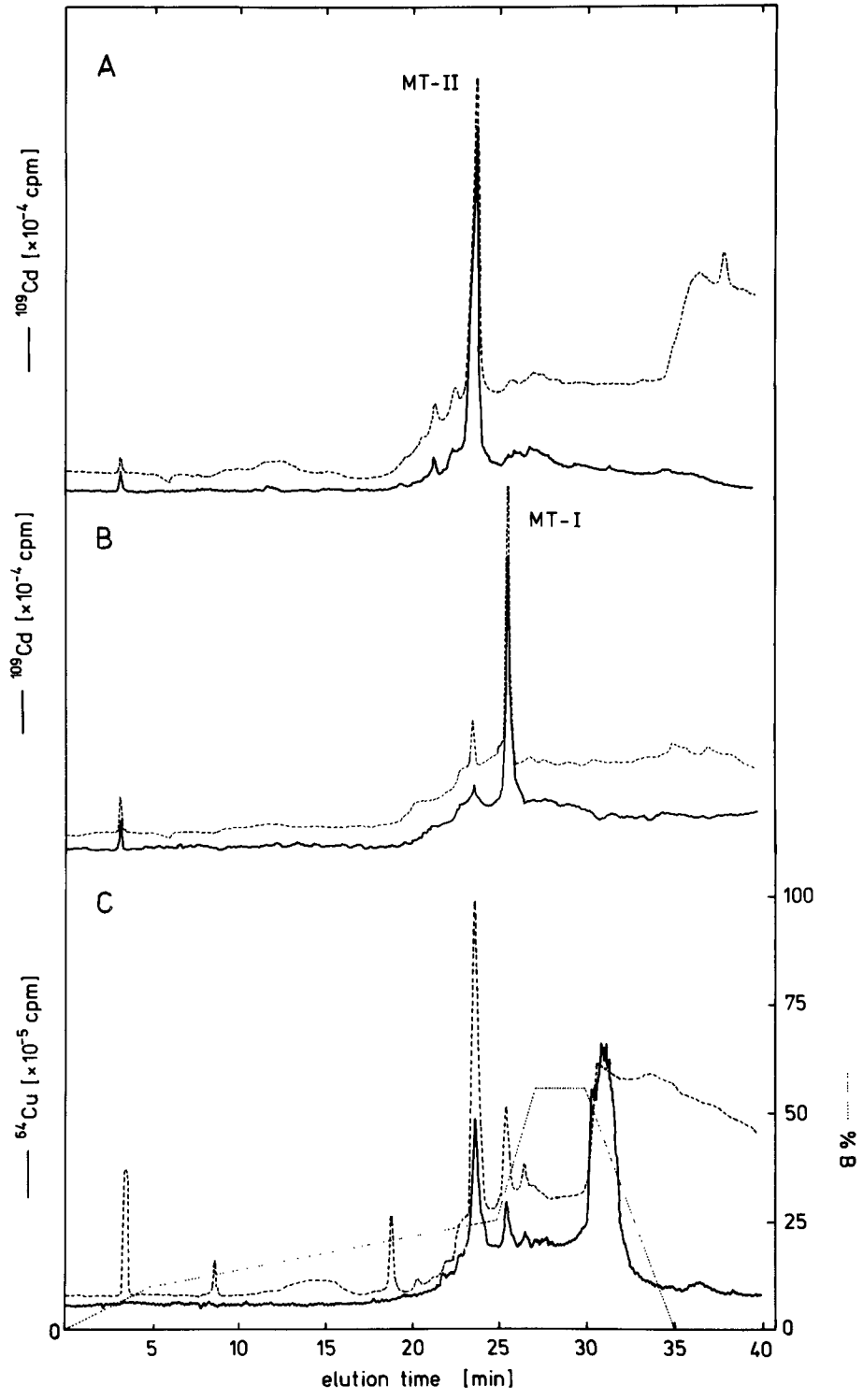

Fig. 2. (A) and (B) Reversed-phase chromatograms of ^{109}Cd-labelled rabbit MT-II and MT-I. Commercially obtained rabbit MT-I and MT-II were radiolabelled by incubation with 0.1 m*M* Cd^{2+} (0.5 MBq ^{109}Cd/mmol Cd), and isolated as described in Experimental. (C) Reversed-phase chromatogram of rat HTC cytosol. HTC cells were incubated in a medium containing 200 μM Cu^{2+} (spiked with 0.5 MBq ^{64}Cu/mmol Cu) for 24 h. Cell cytosol was obtained as described in Experimental. The retention time of HTC MT-I was 25.5 min, and that of MT-II was 23.4 min. The UV detection wavelength was 214 nm.

TABLE II

METALLOTHIONEIN CONTENT, MT-I/MT-II RATIOS AND [^{35}S]CYSTEINE, ^{65}Zn AND ^{64}Cu CONTENT IN RAT HEPATOMA HTC CELLS

Cells were incubated in a medium containing zinc and copper plus radionuclides for 24 h under the conditions given in Experimental. MT concentrations were determined according the Cd-saturation assays described in Experimental. Amounts of radioactivity were converted into absolute amounts by assumed specific activity of the applied media. All data are given as mean values ± S.D.

HPLC conditions	MT-I (pmol/mg cell protein)	MT-II (pmol/mg cell protein)	MT-I/MT-II ratio	Radionuclide (nmol/mg cell protein)	
				MT-I	MT-II
MSE [^{35}S]Cys (+ Zn)	119 ± 9[a]		–	1.88 ± 0.23[a]	
AE ^{65}Zn	38 ± 3	44 ± 3	1.0 : 1.1	0.18 ± 0.03	0.31 ± 0.05
RP ^{64}Cu	51 ± 3	159 ± 6	1.0 : 3.1	0.12 ± 0.01	0.25 ± 0.02
MSE Cd	30 ± 8	106 ± 9	1.0 : 3.5[b]	N.D.[c]	N.D.

[a] Determined as the sum of MT-I and MT-II (MSE).
[b] Determined by amino acid analysis.
[c] N.D. = not determined.

and eluted MT: the value found was 0.83 ± 0.03. The MT recovery after MSE chromatography (TSK G2000 SW and G3000 SW) was also tested directly from HTC cell cytosol MT (mixed I and II isoforms), by ^{109}Cd assay before and after MT elution; the fractional recovery was calculated as 0.95 ± 0.05.

Amino acid analysis

Amino acid analysis of isolated and purified HTC MT-I and MT-II was carried out following Hirs [32]. In Zn-treated cells, MT-I (apparent molecular mass 6628) and MT-II (apparent molecular mass 5959) consist of 66 and 58 amino acid residues, respectively. The differences may be attributed largely to the differences in cysteine residues: the molar ratio Cys:MT was determined as 20:1 and 16:1 for MT-I and MT-II, respectively (Table II). Aromatic residues, such as histidine, tyrosine and phenylalanine, could not be detected. Based on the signals at 220 nm (Fig. 1), Zn-induction resulted in an apparent 1:1 occurrence ratio of MT-I and MT-II isoforms (Table III).

The apparent molecular mass of Cd-induced HTC MT (mixed MT-I and MT-II) was determined as 6109, giving a 1:3.5 occurrence ratio for MT-I and MT-II (Table III). For Cu-treated HTC cells, after Cu-induction and quantification of Cu-MT according to the methods described by Klein *et al.* [27], MT-I and MT-II were determined to occur in a 1:3 ratio.

HPLC of HTC cell cytosol

A number of HPLC experiments were carried out with cytosol samples taken from HTC cells, which were incubated under various conditions. The results demonstrate the potential of the combined use of HPLC and radiotracer techniques. The experiments were carried out in combinations of AE-HPLC with ^{65}Zn, RP-HPLC with ^{64}Cu and MSE-HPLC with [^{35}S]cysteine.

AE-HPLC with ^{65}Zn. HTC cytosol, incubated for 24 h with 200 μM ^{65}Zn-spiked Zn, was preseparated on a preparative TSK G3000 SWG column, to obtain total MT, which eluted at V_e/V_o = 2.05–2.15 (t_R = 33.85–35.50 min). After pooling and desalting (PD-10 columns), MTs were further separated on a DEAE-AE column (Fig. 1): ^{65}Zn-MT-I and -II eluted at 11.5 min (I = 64 mM) and 14.5 min (I = 104 mM), respectively. Based on the signals at 220 nm, MT-I and

TABLE III

AMINO ACID COMPOSITION OF ZINC- AND CADMIUM-TREATED RAT HTC METALLOTHIONEIN

Isolated MT-I and MT-II in Zn- and Cd-treated HTC cells were hydrolysed with 6 *M* HCl at 165°C for 2 h [32]. Values, expressed as residues per molecule, are based on minimum molecular mass as shown. Half-cystine was determined as cysteic acid after performic acid oxidation and hydrolysis [32]. Compositions are given relative to lysine content.

Amino acid	Zn-MT-I	Zn-MT-II	Cd-MT
Aspartate	4.7 (5)	5.0 (5)	4.2 (4)
Glutamate	2.8 (3)	2.6 (3)	2.3 (2)
Serine	5.1 (5)	4.2 (4)	4.3 (4)
Histidine	0.3 (–)	0.3 (–)	0.0 (–)
Glycine	4.9 (5)	4.7 (5)	5.7 (6)
Threonine	4.2 (4)	2.9 (3)	2.8 (3)
Alanine	7.0 (7)	7.0 (7)	7.0 (7)
Arginine	N.D.	N.D.	N.D.
Tyrosine	0.1 (–)	0.1 (–)	0.2 (–)
Cysteine[a]	20.4 (20)	16.1 (16)	19.4 (19)
Valine	0.8 (1)	0.9 (1)	0.5 (1)
Methioneine[b]	1.5 (2)	1.3 (1)	1.4 (1)
Phenylalanine	0.3 (–)	0.3 (–)	0.3 (–)
Isoleucine	1.6 (2)	1.1 (1)	0.8 (1)
Leucine	1.0 (1)	1.1 (1)	0.8 (1)
Lysine	8.8 (9)	8.5 (9)	9.5 (10)
Proline	1.6 (2)	2.1 (2)	0.9 (1)
Tryptophan	N.D.	N.D.	N.D.
Total residues	66	58	60
Minimum mol. mass	6628	5959	6109

[a] Determined as cysteic acid.
[b] Determined as methionine sulphone.
[c] N.D. = not determined.

MT-II were calculated to be present in a 1:1.0 mass ratio. Accounting for the cumulative fractional MT recoveries, Cd-assaying yielded 119 ± 9 pmol MT/mg cell protein in the cytosol of Zn-treated HTC cells.

The ^{65}Zn recovery from the DEAE-AE column was determined as 86 ± 5% (n = 3), of which *ca.* 90% was present in the MT positions. A minor ^{65}Zn fraction was found at t_R = 18.3 min (I = 140 mM), possibly in MT-polymers (Fig. 1). Accounting for the 95% MT recovery, the 86% recovery of ^{65}Zn (injected as ^{65}Zn-MT) in MT positions indicate that *ca.* 20% of the initial ^{65}Zn in ^{65}Zn-MT is lost during the 11–14 min needed to elute both MT-I and MT-II.

Based on the data and conditions described above, and assuming immediate and complete separation of ^{65}Zn and ^{65}Zn-MT, an apparent effective $t_{1/2}$ value for the dissociation of Zn from Zn-MT can be estimated as *ca.* 0.66 h.

During the 24-h uptake period, ^{65}Zn accumulated in the HTC cytosol up to 10.3 ± 1.4 ($\times 10^4$) dpm/mg cell protein. With known cytosolic zinc concentrations (AAS: 1.40 ± 0.18 nmol Zn/mg cell protein) and based on the ^{65}Zn specific activity in the applied solution (75.8 ± 1.2 ($\times 10^3$) dpm/nmol Zn), the fractional cytosolic ^{65}Zn specific activity was calculated as 97%, indicating full equilibrium between cells and solution. Assuming an identical ^{65}Zn/Zn equilibrium in MT,

and based on the ^{65}Zn activities (MT positions) after DEAE-AE chromatography, the molar Zn:MT ratios could be calculated as 4.7 (± 0.9):1 and 7.1 (± 1.1):1 for MT-I and MT-II, respectively; on average, this means 5.9:1 for Zn:MT.

RP-HPLC with ^{64}Cu. HTC cytosol, incubated for 24 h with 200 μM ^{64}Cu-spiked Cu, was directly injected into a C_{18} reversed-phase column (see Fig. 2). At a detection wavelength of 214 nm, the sharp peak at 2.7 min may be attributed to the elution of β-mercaptoethanol; both MT isoforms were eluted between 20% and 30% of buffer B (MT-II at 23.4 min and MT-I at 26.4 min). The chromatogram also showed a relatively large peak at 31.0 min, indicated by both UV detection and ^{64}Cu counting, possibly representing high-molecular-mass cytosolic compounds. The MT signals obtained at 214 nm indicated a MT-I:MT-II mass ratio of 1:2.5. Later assay (*cf.* Klein *et al.* [27]) of MT fractions suggested a ratio of 1:3.1. The column recovery of ^{64}Cu appeared to be 95 ± 5%. The ^{64}Cu presence in MT positions was calculated as only 15% (MT-II) and 7% (MT-I) of the initially injected cytosolic ^{64}Cu.

During the 24-h uptake period, the HTC cytosol accumulated ^{64}Cu up to 63.9 ± 2.9 ($\times 10^3$) dpm/mg cell protein. With known total cytosolic Cu concentrations (AAS: 1.66 ± 0.08 nmol Cu/mg cel protein), and known applied specific ^{64}Cu activity (37.1 ± 1.3 ($\times 10^3$) dpm/nmol Cu), the cytosol and bulk medium was calculated to have reached full equilibrium situation. Applying MT assays under Cu conditions [33,34], MT-I and MT-II could be determined as 51 ± 3 and 159 ± 6 pmol/mg cell protein, with a mean specific Cu presence in MT of 6.4 ± 1.2 mol Cu per mol MT (*cf.* Steinebach and Wolterbeek [33]). The latter data indicate that, under the conditions applied, *ca.* 80% of cytosolic Cu is associated to MT. The 6.4:1 mol ratio obtained for Cu:MT is in sharp contrast to the calculated 2.4:1 and 1.6:1 mol ratios for ^{64}Cu:MT-I and ^{64}Cu:MT-II, respectively (the latter based on applied ^{64}Cu specific activities, the mean ^{64}Cu:MT ratio can be determined as 1.8:1).

These results, and the predominant presence of ^{64}Cu in the large cytosolic (high and low molecular mass) compounds (Fig. 2), strongly suggest precursor functions of one or several cytosolic compounds: these may associate with newly accumulated ^{64}Cu before any further transport of "new" cytosolic Cu into MT takes place. This reasoning indicates that the rate at which ^{64}Cu associates MT will be calculated as reduced, relative to the rate of overall accumulation of ^{64}Cu in the cellular cytosol; the extent of the underlying delay may be related to the concentration (rate of synthesis) and Cu-affinity/capacity of the MT precursor cytosolic compounds.

MSE-HPLC with [^{35}S]cysteine. MT synthesis was induced in [^{35}S]cysteine-treated HTC cells by the simultanuous administration of 200 μM Zn. After MT induction and [^{35}S]cysteine uptake for 24 h, cellular cytosol was obtained and chromatographed on a TSK G2000 SW column. The ^{35}S-labelled MTs co-eluted at V_e/V_o = 1.85 (t_R = 19.80 min). Further amounts of [^{35}S]cysteine could be detected predominantly in the high-molecular-mass fractions (Fig. 3). The column recovery of [^{35}S]cysteine recovery was determined as 89 ± 8% (n = 3), and the fractional presence of [^{35}S]cysteine in MT (relative to injected [^{35}S]cysteine) was 0.39 ± 0.04. After 24 h, 29.5 ± 1.8 ($\times 10^3$) dpm/mg cell protein of [^{35}S]cysteine had accumulated in MT, which, based on the specific activity of the applied bulk [^{35}S]cysteine, the 95% recovery of [^{35}S]cysteine-MT from MSE-HPLC, and the presence of 119 ± 9 pmol of MT per mg cell protein, amounted to 15.8 ± 1.9 mol of cysteine per mol of MT. Based on the amino acid compositions of MT-I and MT-II (Table III) and the unit occurrence ratio of Zn-induced MT-I and MT-II (Table II), the 24-h data for [^{35}S]cysteine may be interpreted as showing almost 90% of the maximum [^{35}S]cysteine incorporation into MT.

In a series of measurements under the experimental conditions described above, the accumulation of [^{35}S]cysteine in both the total cell and in MT was followed during a 48 h [^{35}S]cysteine application period (Fig. 4). The total cell and the MT pool were considered as [^{35}S]cysteine-containing cellular compartments, and labelled C_{TC} and C_{MT}, respectively; the non-MT cellular com-

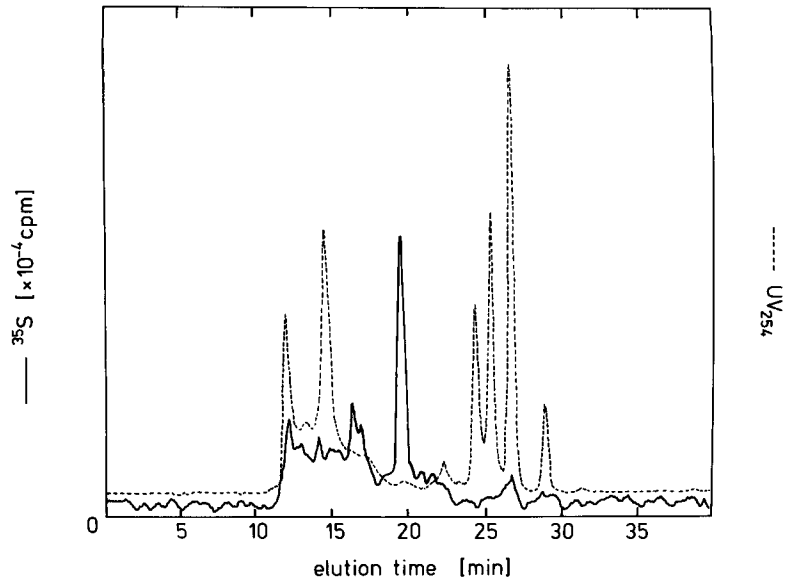

Fig. 3. MSE chromatogram of rat HTC cytosol. Cells were incubated in a medium containing 0.2 m*M* cysteine (spiked with 37 kBq L-[^{35}S]cysteine), supplemented with 25 μM Zn^{2+} for 24 h. Cell cytosol was obtained as described in Experimental. HPLC conditions as described previously. Rat hepatoma MT was detected at V_e/V_o = 1.85 (t_R = 19.80 min). The UV detection wavelength was 254 nm.

partment was labelled C_{nonMT}, so that $C_{nonMT} = C_{TC} - C_{MT}$. In a simple closed three-compartment system, consisting of the culture medium (C_A), C_{nonMT} and C_{MT}, (Fig. 5), the [^{35}S]cysteine data could be processed by fitting bi-exponential functions to the C_{MT} and C_{nonMT} [^{35}S]cysteine time-curves. Table IV presents the derived function parameters, together with the associated

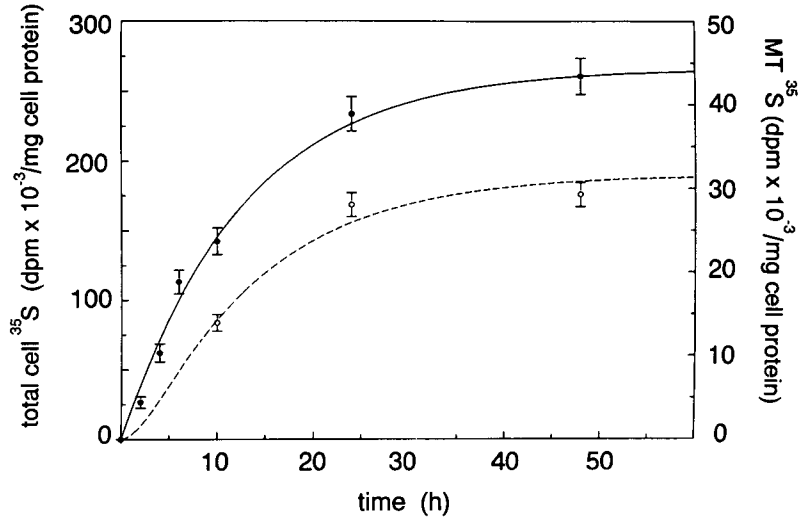

Fig. 4. [^{35}S]Cysteine accumulation in rat HTC cell cytosol and MT from 0 to 48 h. Amounts of [^{35}S]cysteine in cell cytosol were determined by MSE HPLC, measuring on-line ^{35}S counts (in dpm/mg cell protein). Amounts of ^{35}S incorporated in MT were determined by measuring the number of counts in the MT peak area. Solid line, fitted data for total cell ^{35}S; dashed line, fitted data for MT ^{35}S; left ordinate, total cell ^{35}S (closed circles); right ordinate, MT ^{35}S (open circles).

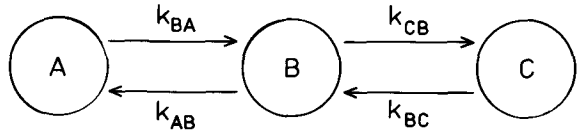

Fig. 5. Compartment analysis of [^{35}S]cysteine-labelled HTC cells: closed three-compartment model. Arrows indicate transfer routes. Rate constants are represented by k_{BA} etc. Compartments: A = culture medium; B = non-MT; C = MT.

first-order rate constants. Note that the g_1 and g_2 values were fitted by simultanuous consideration of both C_{TC} and C_{MT} [^{35}S]cysteine time-curves.

The equilibrium value (at infinite time) for [^{35}S]cysteine incorporated in MT was calculated as 31.66 ± 1.10 ($\times 10^3$) dpm/mg cell protein. After corrections for recoveries and specific activities, this gives a value of 18 ± 2 mol cysteine per mol MT, in perfect agreement with the data shown in Tables II and III, which were derived independently from amino acid analysis and UV spectra.

If the efflux of [^{35}S]cysteine from the MT compartment is taken as an indication of MT breakdown, the k_{BC} constant (Table IV) can be regarded as denoting the fractional rate of MT degradation. From k_{BC} = 0.39 ± 0.15 h^{-1}, the MT half-life can be obtained as 2.0 ± 0.8 h, in reasonable agreement with the MT half-life data of 3–7 h reported by Steinebach and Wolterbeek [22], and 5–6 h reported by Krezoski *et al.* [34].

DISCUSSION

All experiments in the present paper were carried out with rat HTC cells. HTC is a poorly established hepatoma cell line, developed from rat liver tumour, and differs from rat liver parenchymal cells with respect to carbohydrate metabolism, *i.e.* glycolysis and gluconeogenesis [35]. HTC cells were also used previously, because of their rapid proliferation and reproducible behaviour [21,22].

The Zn-, Cu- and Cd-associated MT-like proteins detected in HTC cells were further isolated, purified and characterized (Table II and III). The amino acid composition, apparent molecular mass, and presence (or absence) of specific amino acids showed similar characteristics to the class I MTs reported earlier [36]. The unique aspects of this class of mammalian MTs are the high cysteine content, and the presence of serine and lysine (*ca.* 33% Cys, 14% Ser and 13% Lys of the residues), and the lack of aromatic amino acids, such as histidine, tyrosine and phenylalanine. Furthermore, our results on HTC MTs agree

TABLE IV

FUNCTION PARAMETERS AND RATE CONSTANTS OBTAINED FROM [^{35}S] CURVE FITTINGS

Parameter values generated by fitting a bi-exponential function (see Experimental) to data obtained for the incorporation of [^{35}S]cysteine with time in total cell (solid line) and MT (dotted line in Fig. 4). The A constants are given in dpm ($\times 10^{-3}$)/mg cell protein; the g constants have dimension h^{-1}. Rate constants (h^{-1}) are for a closed three-compartment model: the degradation of MT (k_{bc}), the synthesis of MT (k_{cb}), the uptake of [^{35}S]cysteine from the medium into the non-MT pool (k_{ba}), and the release of [^{35}S]cysteine from the non-MT pool into the medium (k_{ab}). All constants are given as mean values ± S.D.

Curve fit	A_1	A_2	A_3	g_1	g_2
Total cell	−267.5 ± 15.3	−1.26 ± 0.10	266.3 ± 15.2	0.079 ± 0.007	0.436 ± 0.150
MT	−38.69 ± 1.34	−7.03 ± 0.25	31.66 ± 1.10	0.079 ± 0.007	0.436 ± 0.150

Compound	k_{bc}	k_{cb}	k_{ab}	k_{ba}
[^{35}S]Cysteine	0.39 ± 0.15	0.053 ± 0.020	0.0088 ± 0.0010	0.0013 ± 0.0001

with data found for rat hepatocyte MTs (*cf.* Kägi and Kojima [37]).

After treatment of HTC cells with 200 μM Zn, MT-I and MT-II were observed to be present in a 1:1 unit ratio (Fig.1, Table III). This ratio differs from the 1:2 MT-I to MT-II ratio found in rat liver by Lehman-McKeeman and Klaassen [9] during *in-vivo* experiments (1000 μmol Zn/kg applications), notwithstanding the comparable total cellular Zn levels [9,38]. This difference may be explained by the variations in the fractionation of Zn between hepatocytes and HTC cells [38]. Based on the observed total cellular MT levels, the present unit ratio between MT-I and MT-II agrees with results reported by Richards and Steele [19] for *in vivo* experiments, where MTs were determined in chick liver after repeated administration Zn. Furthermore, after Cd induction, HTC cells exhibited MT-I and MT-II in a 1:3.5 ratio (see also the Cu applications, Table III), in contrast to the (*in vivo*) 1:1 ratio in Cd-treated rats [9,39]. Following Richards and Steele [19], these differences may be explained by an excessive uptake of Cd in HTC cells under the 1.0 μM Cd application conditions, compared with the 30 μmol Cd/kg application by Lehman-McKeeman and Klaassen [9].

Based on the on-line Berthold conditions and the applied specific activities for ^{35}S, ^{64}Cu, ^{65}Zn and ^{109}Cd, and taking their respective maximal molar ratios against MT as 20:1, 10:1, 7:1 and 7:1, the absolute limits of detection of MT (defined as three times the noise level, considering baseline separations) were established as 0.4, 0.09, 0.10, and 1.2 pmol, respectively. Practical HPLC-AAS on-line detection of Cd-saturated MTs by Cd measurements yielded absolute detection limits of MT of *ca.* 30 pmol, for 3 ml/g tissue dilutions and 100-μl HPLC injections [9,16]. Summer and Klein [14] compiled sensitivity data for MT detection, and discussed detection limits ranging from 10^{-9} to 10^{-15} mol, the lower values primarily obtainable by RIA/ELISA procedures, thereby linking optimal selectivity and sensitivity. Based on the above, and although probably inadequate for general MT determinations in body fluids [14], the present sensitivity may be regarded as perfectly suited for determinations of basal tissue MT levels.

The MT recovery after PD-10 desalting was established as *ca.* 80%. This relatively low figure may be attributed to the early cut-off of the MT fractions, primarily meant to achieve optimal salt separations. All other MT recoveries were *ca.* 95%, irrespective of the mode of MT isolation (AE, RP or MSE). The latter values are in close agreement with data reported elsewhere [16–19], irrespective of the various differences in combinations of columns, buffers, and other chromatographic conditions.

Careful monitoring of the recoveries of both ^{65}Zn and MTs during AE chromatography (Fig. 1) indicated that *ca.* 20% of the ^{65}Zn was lost from MT during elution. The derived half-life of *ca.* 0.66 h for the Zn-MT complex agrees with results given by Krezoski *et al.* [34], who reported a half-life of 1 h Zn-MT in Chelex-treated Ehrlich cells. The relatively fast removal of Zn from MT is consistent with the idea of Zn being kinetically labile in the thiolate clusters of the protein [13,40]; recent studies also showed that Zn may undergo facile and rapid exchange within and between metal-binding clusters and between molecules [41,42]. The importance of the determination of rates of metal removal from MT may be illustrated by suggestions that the more rapid release of Zn from MT-I causes MT-I to be degraded more rapidly than MT-II [43], thereby coupling MT degradation to the stabilities of their metal complexes.

The values for the stability constant for the Cu-MT complex are generally reported to be up to six orders of magnitude higher than for Zn-MT [44]. Although this may help explaining the high ^{64}Cu recovery during RP liquid chromatography (95%), the small fractional ^{64}Cu presence in MT (relative to total cytosolic ^{64}Cu) indicates the importance of other cytosolic compounds in complexing newly imported Cu (^{64}Cu). Because ^{64}Cu reached only 28% of the ultimate steady-state level in cytosolic MT in the 24-h uptake period, the predominant presence of ^{64}Cu in the non-MT cytosolic compound(s) (Fig. 2) may indicate the operation of one or several transfer or

precursor compounds. This line of reasoning is supported by results obtained by Freedman *et al.* [45], who suggested that Cu entering the cell would be rapidly complexed by reduced glutathione (GSH), but stored in MT after completion of the necessary transfer processes. For copper-resistant HAC_{600} cells, Freedman *et al.* [45] reported that only 0.28 h was needed for ^{67}Cu to reach 50% of its steady-state value in MT, but in wild type HAC cells more than 6 h were needed to reach equilibrium. The present data for HTC cells suggest much slower kinetics, which, following Freedman *et al.* [45], may be partly due to differences in levels of cytosolic MT relative to GSH, but which may also be attributed to the limited capacity of HTC cells to accumulate Cu [33].

During the 48-h accumulation of [^{35}S]cysteine (Fig. 3), measurements of ^{35}S incorporation were carried out in total cell and in isolated MT fractions (see Steinebach and Wolterbeek [38] for steady state of MT levels after 24 h for 5–200 μM Zn applications). As the theories involved in compartmental analysis rule that, in all compartments considered, similar g-values should be expected [30,31], fitting routines were adapted to permit simultanuous consideration of ^{35}S-countings in both total cell and MT curves. As a result, the g-values for the MT curve could be largely derived from the more easily established total-cell ^{35}S accumulation curve (Fig. 3). This, in turn, allowed reliable estimates of function constants determined from only a limited number of recordings of ^{35}S in MT (Table IV). The application of compartmental analysis permitted the determination of actual rates of MT degradation under conditions of simultanuous MT synthesis [22]. Note that the losses of ^{35}S were considered to represent breakdown of MT. The MT half-life of *ca.* 2 h is considerably shorter than generally reported for MT biodegradation either *in vivo* or *in vitro* [39,43], but agrees with MT rates of degradation given by Steinebach and Wolterbeek [22] and Krezoski *et al.* [34]. The presented half-life value at which ^{65}Zn is lost from HTC MT during AE chromatography (Fig. 1) is in line with the half-life value of 1 h found by Krezoski *et al.* [34], indicating that loss of Zn from MT may occur very rapidly, especially when considering reports from other authors [46,47], who suggested that MT degradation may be rate-limited by the kinetics of MT–metal dissociation.

CONCLUSION

These results have established the MT characteristics of the metal-accumulating cytosolic protein in HTC cells, and indicate the applicability of the combined use of HPLC techniques and (on-line) radioactivity monitoring. The use of radiotracers was shown to permit the determination of newly imported metal ions (^{64}Cu/RP-HPLC experiments), or monitor the actual rates of MT–metal dissociation (^{65}Zn/DEAE-AE experiments), which were both shown to be of potential advantage in metabolism studies. Finally, the work has shown how the combined application of ^{35}S-labels and compartmental analysis can be used to determine the rates of MT degradation, even under ^{35}S accumulation conditions.

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