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Apometallothionein in rat liver

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ABSTRACT

The identification of apometallothionein (AMT) in rat liver by reversed-phase high-performance liquid chromatography (RP-HPLC) after gel permeation was realized in experiments performed both *in vivo* and *in vitro*. The reliable assignment of the corresponding AMT peak permitted the detection and determination of AMT in different groups of experimental and control rats. In all animals studied (more than 100 rats), AMT was always present in amounts higher than that of metallothionein (MT) or compatible with it. Induction of MT synthesis by $CdCl_2$ subcutaneous injections decreased the AMT level and increased the MT level, but nevertheless the amount of AMT still remained relatively high.

INTRODUCTION

Metallothionein (MT), isolated first from horse kidney and described as a Cd- and Zn-containing protein, is thought to play a major role in intracellular zinc metabolism [1]. MT is a small protein ($M_r \approx 6000$) built up from 61 amino acids, including 20 cysteine residues with sulphhydryl groups capable of binding up to seven atoms of metals (Zn, Cd, Hg, etc.) per molecule [2].

The synthesis of cellular MT may be induced by numerous factors, such as metals (Zn, Cd, Cu) [3], glucocorticoids, interleukin I, cathecholamines, oxidative stress and infection [4]. There is evidence that MT donates zinc to Zn-dependent apoenzymes at least *in vitro* [2]. Thus MT in tissues is involved in the uptake, storage and intracellular distribution of Zn and Cu. Apometallothionein (AMT, thionein) represents the metal-free form of MT. Up to now AMT has been obtained only by *in vitro* experiments through demetallation of MT either by other ligands or by acidification with strong acids [5]. Usually it is accepted that AMT is first synthesized in the organism, followed by complexation with metals in the cytosol [6]. Nevertheless, it has been mentioned that AMT is not found in appreciable amounts at least among mammals [1].

In a recent study [7], we found that in the process of purification of (Cd, Zn)-MT from rat liver by gel permeation, the protein concentration of the MT-containing fraction is higher than that of MT, determined by HPLC applied after

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gel permeation. In addition, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) experiments performed after gel permeation have shown that there is only one band of protein observed, corresponding to MT. Taking into account the relatively low resolution of the method, we assumed that probably AMT is also present together with MT in the sample.

In this connection several important problems arise, namely, whether in normal animals appreciable amounts of free AMT exist or only MT, if AMT is normally present, what the concentration ratio of AMT to MT is and which form is in excess, and how the AMT level and the MT/AMT ratio are influenced following stimulation with metals.

The answers to these questions, which were the aim of this work, could help to clarify many other problems related to the biosynthesis of MT, to the role of AMT in the mechanism of bonding of bio- and toxic metals and of regulation of the biometal homeostasis. This could also help to discriminate between the possible differences in the biological action of AMT and MT, which has hardly been discussed so far.

From this point of view, the hypothesis of the possible relationship between the concentration level of AMT/MT and arterial hypertension [8] could also be of interest.

EXPERIMENTAL

Chemicals

All reagents used were of analytical-reagent grade from Merck (Darmstadt, Germany), if not specified otherwise. Aqueous solutions were prepared with deionized water (18 MΩ) obtained from a Milli-Q reagent water system (Millipore, Milford, MA, USA).

Animals

Ten Wistar–Kyoto rats were used as the experimental group, another ten animals of the same origin and parameters were the control group and two other groups (sixteen animals each) were used in the experiments with $CdCl_2$ stimulation shown in Figs. 5 and 6. The animals were 12 weeks old with initial body mass of 198–237 g. They were housed individually in stainless-steel cages in an animal facility with a 12-h dark–light cycle, had a standard briquette chow with normal dietary NaCl (4.4%) and KCl (0.95%) and tap water was offered *ad libitum*. In order to induce MT synthesis in the experimental rat livers, subcutaneous injections were made once daily (2.0 mg of $CdCl_2$ in physiological solution per kg body mass) for 3 days. The animals were killed by decapitation 24 h after the last injection.

Sample preparation

All operations were carried out at 4°C under nitrogen. The liver preparation involved homogenizing the tissue with a PTFE homogenizer (8–10 strokes) as described by Suzuki [9] in 3 volumes of 0.1 *M* Tris–HCl buffer (pH 7.4) containing glucose (25 m*M*). The homogenate was centrifuged at 170 000 *g* for 60 min and the supernatant (4.0 ml) was used further. A Sephadex G-75 column (1000 mm × 24 mm I.D. and V_o = 130 ml, V_t = 407 ml) from Pharmacia (Uppsala, Sweden) was used for size-exclusion chromatography. Elution was carried out with 50 m*M* Tris–HCl buffer (pH 6.8) at a flow-rate of 1.0 ml min^{-1} [9].

The molecular mass (M_r) of proteins was estimated using the following standard molecular-mass marker proteins (Pharmacia) as references: chymotripsinogen, 25 000, K_d = 0.29; ribonuclease type A, 13 700, K_d = 0.48; and insulin, 5000, K_d = 0.78 (Novo, Bagsvaerd, Denmark). MT was identified as a peak with K_d = 0.65. The absorbance of the eluate at 254 nm (A_{254}) was continuously recorded using a flow cell and a UV detector (Pharmacia).

Fractions of 5.0 ml were collected. All fractions from No. 30 to 40, corresponding to the MT peak, were combined and concentrated under nitrogen on an Amicon (Danvers, MA, USA) YM2 membrane to a final volume of 10 ml. The purity of the protein thus obtained in the range M_r 10 000–14 000 was checked by SDS-PAGE according to Laemmli [10], under which conditions only one band was observed, corresponding to M_r 14 000.

The protein content of the concentrate was determined by the modified method of Bradford [11]; the calibration graph for the protein content was prepared with standard MT-1 substances (Sigma Chemie, Deisenhofen, Germany). The Cd and Zn contents were determined by electrothermal atomic absorption spectrometry (ETAAS) on a Perkin-Elmer Zeeman 5000 apparatus with an HGA-500 graphite furnace, AS-40 autosampler and ASDS-10 data station.

HPLC experiments

Aliquots of the concentrated solutions (200 μl) were used for the HPLC determination of MT and AMT. HPLC was performed on a semi-preparative μBondapak C_{18} column (300 mm × 7.8 mm I.D., 5 μm particle size) obtained from Waters (Milford, MA, USA). The eluent system consisted of solution A [Tris–HCl buffer (pH 6.8)] and solution B [solution A–acetonitrile (40:60, v/v]; the elution rate was 1.25 ml min^{-1}. The buffer gradient is shown in Fig. 3. A UV detector set at 220 nm (Model 2141 variable-wavelength monitor; LKB, Bromma, Sweden) was used for monitoring. The samples were injected manually.

The HPLC system was used also for amino acid analysis of the two isoforms of MT. The procedure has been described elsewhere [7].

The isoforms MT-1 and MT-2 were designated according to their order of elution: MT-2 elutes before MT-1 at pH 6.8 [12].

Preparation of AMT

The determination of AMT in biological samples by HPLC needed a preliminary preparation of AMT standards. The latter were obtained from MT purified by gel permeation and HPLC. Two different approaches were used for the preparation of AMT by removal of metal ions from MT. In the first, the metal ions were removed using the strong complexing agent EDTA. For this purpose aliquots of 1 ml of the combined concentrates were mixed with 100 μl of EDTA solution of five concentrations, $C_{MT} = 2.5 \cdot 10^{-5}$ *M*.

In order to check whether the presence of EDTA influences the HPLC determination of AMT, control experiments were performed, in which the MT-containing samples were dialysed against EDTA in a large excess (EDTA : MT molar ratio = 21:1). Cellulose tubing from Sigma (St. Louis, MO, USA) was used, separating proteins with $M_r \geq 2000$. In order to remove the last amount of EDTA and its metal complexes, the MT sample was then dialysed against pure deionized water for 48 h with three changes of the deionized water.

The second approach for AMT preparation was based on the dissociation of MT in strongly acidic solutions. For this purpose, the MT-containing samples were treated with HCl to a final pH value of 1.0, where according to literature data [5] MT is completely dissociated. Results were also obtained in another way, by treating the samples mixed with EDTA (EDTA : MT = 100 : 1) with HCl to final HCl concentrations as follows: (a) 0; (b) 0.012; (c) 0.017; (d) 0.023 and (e) 0.186 *M*.

The formation of AMT in the two approaches used was followed by measuring the UV absorption of the samples. They were scanned after the addition of EDTA or HCl in the range 224–300 nm in 15 min on a Beckman DU-50 spectrophotometer (1-cm cell). The spectra did not change during the next 12 h and the solutions were kept at 4°C.

The AMT samples obtained from MT through dialysis and treatment with HCl were both used as standards in the HPLC experiments.

SDS-PAGE

The protein purity was monitored by SDS-PAGE [13]. The gels were stained with Coomassie Brilliant Blue (Serva Blue R) and destained in 20% methanol–10% acetic acid. The electrophoresis was carried out at 200 V.

Calibration of the gels was performed using polypeptide molecular-mass markers from Pharmacia: intact myoglobin, 17 200; myoglobin I and II, 14 600; myoglobin I, 8240; myoglobin II, 6380; and myoglobin III, 2560.

RESULTS AND DISCUSSION

Following $CdCl_2$ stimulation and determination of Cd, Zn and thionein, the results indicated that the HPLC-separated MT form was $(Cd_{5.1}Zn_{1.9})$-MT.

The MT from animals stimulated with $CdCl_2$ was separated by HPLC and treated with increasing EDTA concentrations. The absorbance of the samples thus prepared was measured in the UV region.

The spectra obtained at different EDTA:MT molar ratios are shown in Fig. 1. The results show that higher EDTA:MT ratios lead to a decrease in the absorbance at both 224 and 254 nm, where Zn-MT and Cd-MT absorb, respectively (curves b–e). A further increase in this ratio results in higher absorbance at 235 nm, where cysteine thiolate absorbs [14,15] in slightly alkaline media ($\Delta\varepsilon_{235} = 4500\,l\,mol^{-1}\,cm^{-1}$). The peak at 235 nm (curves f and g) results from the formation of a relatively high concentration of AMT at pH 8.6.

The presence of EDTA in the sample does not affect the UV spectra, as shown by an experiment in which EDTA was removed by dialysis against water. The UV spectrum of that dialysate does not differ from those shown in Fig. 1 (curves f and g), indicating that no interaction takes place between the chelating agent and AMT.

The fact that the absorbance at 235 nm is connected with free, non-coordinated thiolates in alkaline media was confirmed by addition of HCl. Small volumes of concentrated HCl were added to the buffered sample with an EDTA:MT ratio of 100, corresponding to Fig. 1f. The UV spectra of these acidified samples are shown in Fig. 2.

The first small amounts of HCl added (Fig. 2b and c) did not affect the pH of the buffered sample, whereas higher HCl concentrations overcame the capacity of the Tris–HCl buffer and shifted the pH to more acidic values. As a result,

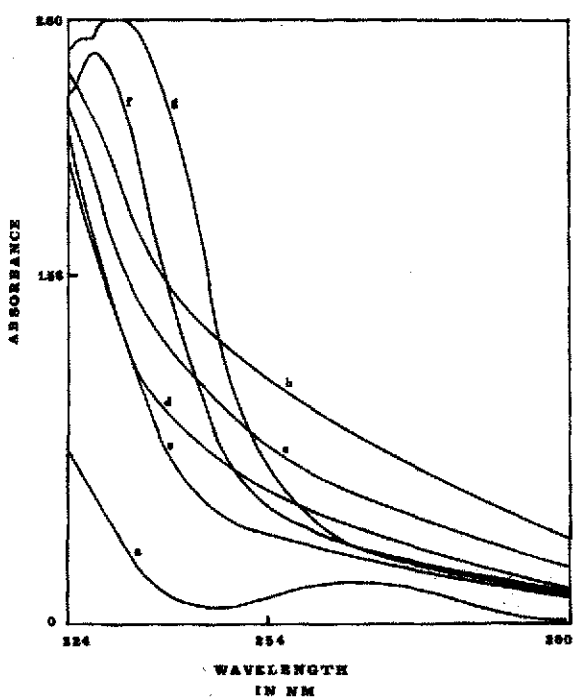

Fig. 1. UV spectra at different EDTA:MT molar ratios. (a) MT from rats not treated with $CdCl_2$; EDTA:MT molar ratio = (b) 0; (c) 0.42; (d) 4.2; (e) 21; (f) 100; (g) 300. pH = 8.6 for all samples.

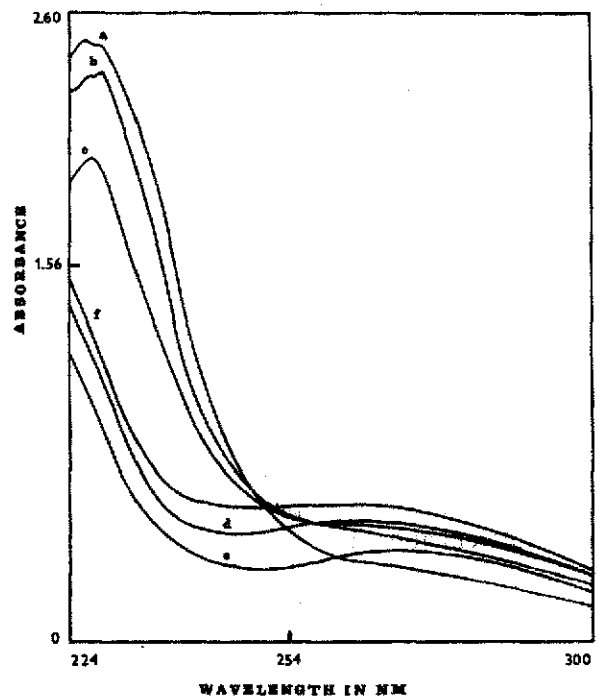

Fig. 2. UV spectra of the samples with an EDTA:MT molar ratio of 100 after addition of HCl. (a) No HCl added, pH 8.6. Total final concentration of added HCl in the sample: (b) 0.012; (c) 0.017; (d) 0.023; (e) 0.186 *M*. (f) Sample containing only MT and HCl, pH 1.

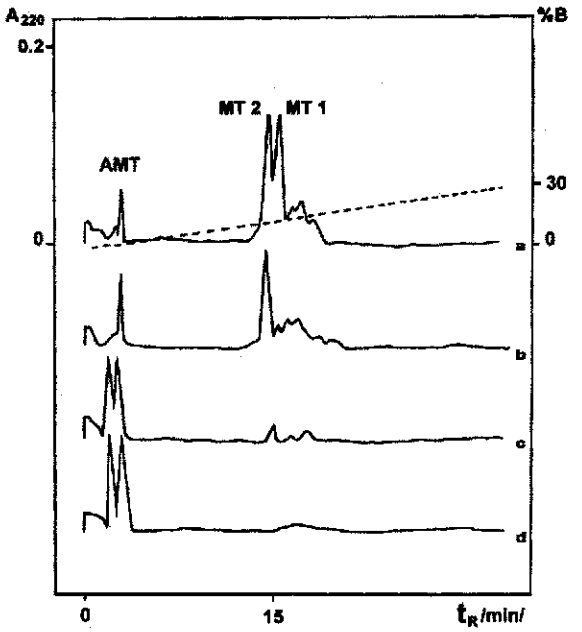

Fig. 3. HPLC of MT after EDTA treatment: (a) MT = $3.9 \cdot 10^{-5}$ *M*, no EDTA present; (b) MT = $3.9 \cdot 10^{-5}$ *M*, EDTA:MT = 2.8; (c) MT = $1.9 \cdot 10^{-5}$ *M*, EDTA:MT = 26; (d) MT = $7.7 \cdot 10^{-6}$ *M*, EDTA:MT = 104.

protonation of the free thiolate groups occurred and the absorbance at 235 nm diminished (Fig. 2d and e), owing to the lower absorption coefficient, ε, of RSH than that of RS^- [14,15].

The most interesting results were obtained by HPLC (Fig. 3) of the same samples as in Fig. 1, except that EDTA was not removed from the sample. The HPLC retention times (t_R) were statistically evaluated by Student's *t*-test, $P < 0.05$. The corresponding elution times in minutes and S.D. values were MT-2 14.37 ± 0.06 (n = 196) and MT-1 15.30 ± 0.11 (n = 196). The results show that treatment with relatively small amounts of EDTA (Fig. 3b) led to a strong decrease in the MT-1 peak, with MT-2 remaining almost unaffected. Larger amounts of EDTA (curve c) reduced both MT-1 and MT-2, leaving only small amounts of MT-2 in the sample. At still higher EDTA concentrations (curve d), MT-1 and MT-2 were both completely transformed into AMT.

Thus, in the course of AMT identification through EDTA treatment of MT, we confirmed the conclusion of Winge and Miclossy [16, 17] that the metal in MT-2 is bonded more strongly than in MT-1, and is removed first from the MT-1 when a mixture of MT-1 and MT-2 is treated with an EDTA solution.

The decreases in the MT-1 and MT-2 peaks were accompanied by the simultaneous appearance of two new peaks with retention times of 1.64 ± 0.16 min (n = 50) and 2.37 ± 0.15 min (n = 50). The first peak, however, disappeared after dialysis against water, and was ascribed to the metal complexes of EDTA (Fig. 4a). The peak with $t_R \approx 2.3$ min was assigned to AMT, obtained through demetallation of MT.

The metal content of the fraction with $t_R \approx 2.3$ min obtained after EDTA treatment and dialysis was studied by ETAAS. No Cd was found in the sample, but traces of Zn were identified. Nevertheless, we consider that probably the very low and variable Zn concentrations observed resulted from sample contamination. The assignment of the AMT peak was confirmed by HPLC experi-

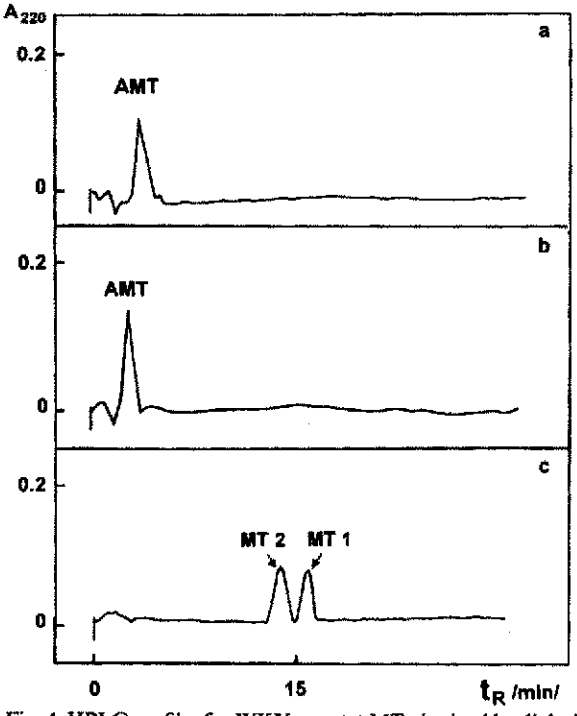

Fig. 4. HPLC profiles for WKY rats. (a) MT obtained by dialysis of MT first against EDTA and then against water; (b) AMT obtained after MT treatment with HCl, pH 1; (c) AMT ($2.5 \cdot 10^{-5}$ *M*) + $CdCl_2$ ($1.5 \cdot 10^{-3}$ *M*).

ments with the samples acidified with HCl (Fig. 4b). In those experiments, the disappearance of the MT-1 and MT-2 peaks was connected with the formation of only the peak corresponding to AMT (t_R = 2.16 ± 0.06 min) (n = 50).

The identification of the peak with $t_R \approx 2.3$ min in Figs. 3 and 4 as being due to AMT is supported by other experiments. AMT obtained from WKY rats was treated *in vitro* with $CdCl_2$ and the peak was monitored by HPLC. The results clearly demonstrated that incubation with $CdCl_2$ reduced the peak with $t_R \approx 2.3$ min at the expense of the two corresponding MT peaks (Fig. 4c).

The data for the identification of AMT in WKY rats under control conditions and after induction with $CdCl_2$ *in vivo* are shown in Fig. 5. In these experiments, the concentration of MT in the control animals was below the detection limit, so we were not able to detect MT. By increasing the total amount of MT taken for the analysis by the use of larger aliquots of the concentrated solutions, and at the same time increasing the sensitivity of the instrument, the MT concentration was determined successfully. It must be emphasized, however, that even with the lower sensitivity AMT was detected in the control group (Fig. 5, t = 0). The MT stimulation *in vivo* with $CdCl_2$ led to a decrease in the AMT peak and enhancement of the peaks corresponding to MT-1 and MT-2 (Fig. 5, t = 3 days).

The presence of a relatively large amount of AMT in the control WKY rats was confirmed independently by SDS-PAGE (Fig. 6). In these experiments, AMT (Fig. 6c) and MT (Fig. 6b) were first purified by gel permeation and HPLC.

As can be seen, AMT and MT are not detected separately by SDS-PAGE, as is realized by HPLC. This may be due to the different mechanisms of separation on which the two methods are based: mainly molecular mass in electrophoresis and hydrophobic properties in HPLC. The small difference in molecular mass between AMT and MT together with the relatively low resolution of the SDS-PAGE method may be responsible for this effect. In addition, the possibility of a denaturing action of the gel matrix also could not be excluded. As for HPLC, the replacement of a metal ion in MT with a proton to form SH

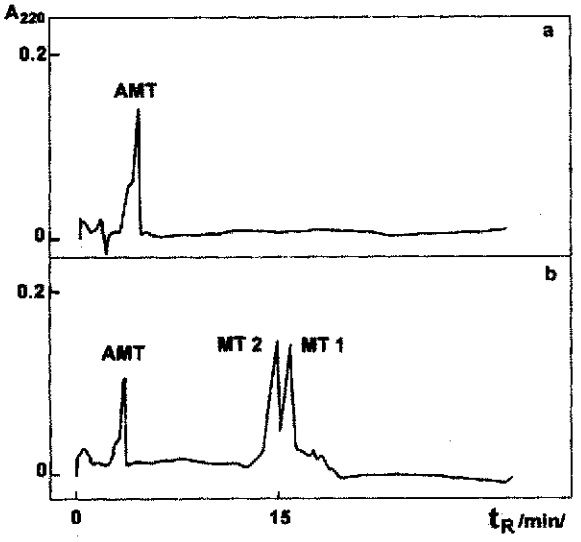

Fig. 5. HPLC pattern of samples from WKY rat liver. (a) Untreated control rats; (b) rats stimulated with $CdCl_2$ injections for 3 days.

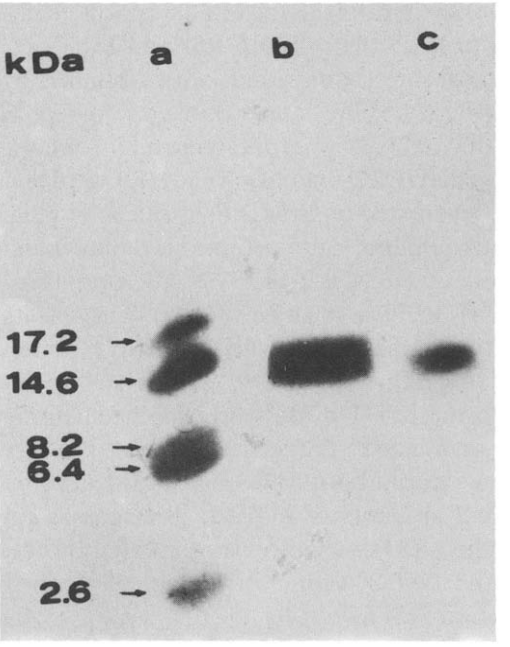

Fig. 6. Polypeptide electrophoresis. (a) Molecular-mass markers; (b) MT from rats after $CdCl_2$ injections for 3 days; (c) AMT from untreated control rats.

leads to a strong increase in the hydrophilic properties of the AMT molecule.

On the other hand, the great difference between the t_R values of AMT and MT in HPLC reveals that a substantial change in the secondary or tertiary structure of AMT with respect to MT took place in the process of demetallation.

Another interesting problem arising from this work is why the isoforms of AMT are not detected in the HPLC experiments. As AMT could be regarded as a precursor-like substance in the formation of MT, leading to the isoforms MT-1 and MT-2, it seems that probably analogous isoforms of AMT must correspond to these. The absence of separate peaks on the HPLC trace, however, may be due to the very similar structure and hydrophilic properties of such forms, making their efficient resolution impossible.

The results obtained in this study have shown that AMT was observed in all the control WKY animals studied and in higher concentration than MT. After stimulation with $CdCl_2$, its concentration decreased, but AMT was still present in relatively large amounts after the formation of additional amounts of MT-1 and MT-2.

In a previous study on the relative MT content in spontaneously hypertensive rats as compared with WKY rats [7], we also found a considerable amount of AMT in all rats from both groups of animals. That result means that AMT is normally always present in the liver cells and it co-exists there with MT even at an increased metal level. Evidently the liver cells maintain a homeostatic level of AMT to be able to react when the metal concentration exceeds a threshold value.

Hence the existence of AMT in the rat liver cells has been firmly established by *in vivo* experiments, although for a long period serious doubts existed as to whether AMT is really present there in appreciable amounts [1]. That experimentally established fact means that the formation of MT should be always preceded by a synthesis or release of AMT. In addition, at least part of the AMT could result also from removal of metal ions from MT in processes of metal transfer from MT to some apoenzymes. Hence, AMT and MT form a well balanced pool, the capacity of which is dependent on the metal ion homeostasis and at the same time is among the factors that, influence this homeostasis. Obviously, the next problem to be studied in this field is to elucidate whether in cytosol AMT, MT and partially saturated MT co-exist simultaneously.

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