NUCLEAR ^{115}CADMIUM: UPTAKE AND DISAPPEARANCE CORRELATED WITH CADMIUM-BINDING PROTEIN SYNTHESIS

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SUMMARY

The intracellular distribution of ^{115}cadmium was determined following a pulsed exposure to the metal. The uptake and disappearance of label from rat liver nuclei was correlated with the appearance of a cytoplasmic Cd-binding protein. By coupling *in vivo* - *in vitro* experiments it was shown that unspecifically bound cadmium is free to enter the nucleus while specifically bound cadmium remains in the cytoplasm.

INTRODUCTION

It now appears that animals respond to short term sublethal levels of several heavy metals (Hg, Cd, Zn, Cu) by producing specific cytoplasmic metal-binding proteins (1-5). Neither the mechanisms which produce and regulate this response, nor the precise roles metals play in mediating it are clear.

Cadmium, the most toxic element in this group (6, 7), is also the most effective agent for eliciting the production of a specific Cd-binding protein in the liver of challenged animals (8, 9). The synthesis of CdBP has been shown to be sensitive to transcriptional inhibitors (8, 9). Selective transcription of DNA in eukaryotic cells is thought to involve the activation of particular genes by extracellular effectors (16, 17). Steroid hormones are well known effectors which bind to cytoplasmic proteins (18), move into the

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Abbreviations:CdBP Cd-Binding Protein; SSC/100, 1.5 mM NaCl, 0.15 mM Na-citrate, pH 7.0.

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nucleus and activate transcription. In this paper, we study the nuclear movement of cadmium as it correlates with the induction of CdBP and its appearance in the cytoplasm. We also consider the possibility that cadmium either directly or in association with other molecule(s) acts as an effector in the induction of CdBP.

MATERIALS AND METHODS

Experiments were carried out using laboratory bred Fisher strain rats that had free access to Purina Rat Chow and water. Experimental animals were injected intraperitoneally with labeled ^{115}Cd (New England Nuclear) and

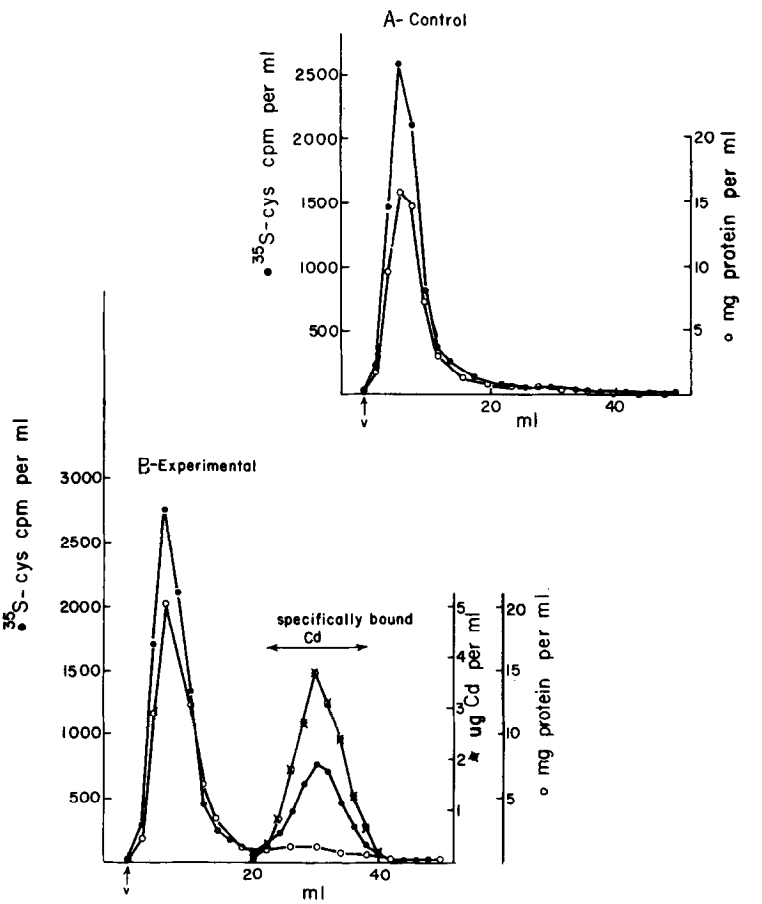

Fig. 1. Induction of CdBP. A & B, animals were given two injections at 0 and 48 hours each of either 7.5 μCi ^{35}S-cystine, 5 μmoles of Cd in 2 ml water (experimental) or 7.5 μCi ^{35}S-cystine in 2 ml water (control); all animals were sacrificed after 96 hrs.

unlabeled cadmium chloride, and/or labeled ^{35}S-Cystine (New England Nuclear). Animals were anesthetized with ether and exsanguinated by cardiac puncture, livers removed, washed 4-5 times with 0.14 M NaCl, minced with a blade and washed an additional 4-5 times in 0.14 M NaCl. Approximately 15 g of tissue were added to 50 ml of 0.25 M sucrose, "TKMC" buffer pH 7.4 (0.05 M Tris, 0.005 M $MgCl_2$, 0.003 M KCl, 0.0002 M $CaCl_2$) and homogenized as described (14, 15). Following centrifugation (700 g for 10 min), a crude nuclear pellet and supernatant were obtained for the subsequent isolation of nuclei and fractionation of cytoplasmic Cd-binding proteins respectively. The pellet was resuspended in 25 ml of 0.25 M sucrose in "TKMC" to which 0.1% Triton X-100 had been added, and otherwise treated essentially as described for nuclei isolation (14, 15) except for the addition of 0.1% Triton X-100 and "TKMC" buffer to all solutions beyond the first step (crude pellet). Purified nuclei were washed (0.25 M sucrose "TKMC"-0.1% Triton X-100) and suspended in 0.01 M Tris, 0.005 M $MgCl_2$, 0.0002 M $CaCl_2$ without Triton X-100. An aliquot of the supernatant (700 g) was used in gel filtration experiments carried out at 0-4° C in a water jacketed column (1.65 cm x 63 cm), packed with fine Sephadex G-50 (Pharmacia) and equilibrated with 0.02 M Tris-HCl pH 8.6 containing 0.02% sodium azide. In the ^{35}S-cystine experiments, the supernatant was recentrifuged (100,000 g for 1 hr) prior to gel filtration. For scintillation counting, 0.2 ml of sample (nuclei or cytoplasmic proteins) were dissolved 1:5 (v/v) with a tissue solubilizer (Eastman) to which 10 ml of scintillation cocktail were added and counted in a Beckman Model LS-1000 counter with an efficiency of about 68% for ^{115}Cd and 60% for ^{35}S [The reported counts are not adjusted for efficiency]. Protein was measured by the modified Lowry method (10) and DNA by A_{260} in 8 M Urea, 0.05 M Tris, 1% sodium dodecyl sulfate pH 8.0. All protein elution profiles are the same as that shown in Fig. 1 except in Fig. 2B where protein denaturation (at 37° - 30 min) may account for the broadening of the peak. Total cadmium (Fig. 1B) was measured by flame atomic absorption with a Perkin-Elmer 360 atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

Twenty four hours following cadmium challenge, the cytoplasmic profile showed metal bound to a cysteine-rich protein fraction, similar to ones described by other workers as metallothionein[1] (11, 12). For characterization purposes, two consecutive (unlabeled) cadmium injections were given to enhance the uptake of labeled cysteine prior to sacrificing animals (Fig. 1B). An 88% increase in the amount of cysteine in this fraction in the experimental animal, as compared to the control animal, was observed. It was also determined by using radioactively labeled ^{115}cadmium, that more than 90% of the metal was bound to the cysteine-rich fraction 24 hrs after injection (Fig. 3B). However, early after exposure (1 hr after cadmium

[1] There is confusion in the literature concerning the resolution of metallothionein into one or more active species; therefore, we prefer to call our impure fraction specific cadmium binding protein (CdBP), rather than metallothionein.

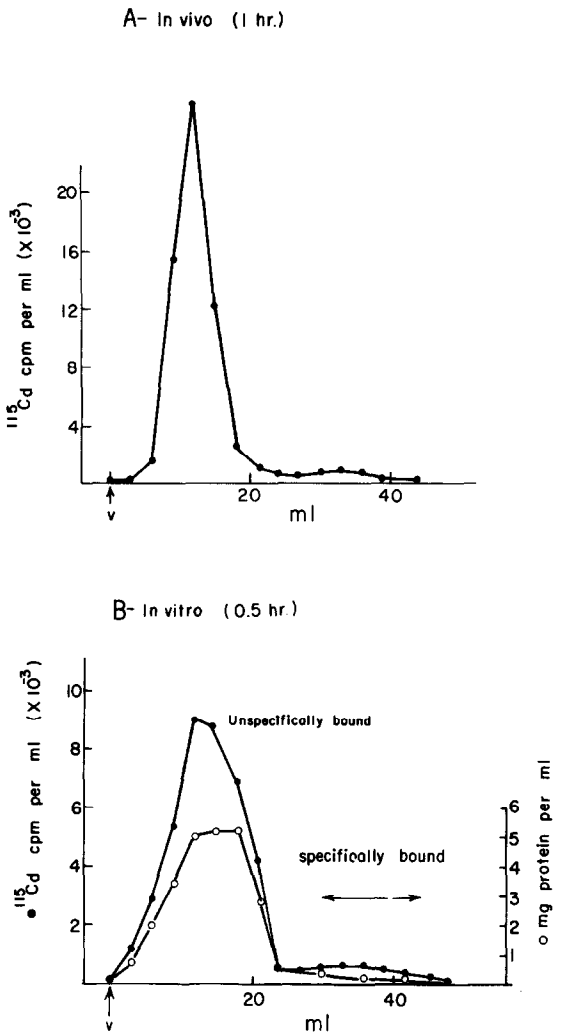

Fig. 2. In vivo and in vitro Cd-binding. Cytoplasmic profiles (Sephadex G-50) of protein and ^{115}Cd after treatment as described in the legend to Table IA, experiments 1 and 3.

injection), the cytoplasmic profile shows the metal to be unspecifically (loosely) bound (Fig. 2A) to large (MW > 30000) proteins indicating that CdBP is essentially absent one hour after injection. This same profile is observed in vitro after adding cadmium to control homogenate and incubating for 30 min at 37° C (Fig. 2B). The level of unspecifically bound cytoplasmic metal decreases with time and, at 24 hrs, is less than 8% (Fig. 3A). In contrast, the level of specifically bound metal increases progressively

with time such that at 24 hrs, more than 90% is associated with CdBP (Fig. 3B). Moreover, there is a strong correlation between the binding of cadmium by this protein fraction and the uptake and disappearance of nuclear bound cadmium (Fig. 3B), indicating that the CdBP is sequestering metal from the internal environment of the cell. This supports the view proposed by others that CdBP has a protective detoxifying role (18, 9).

In the absence of CdBP (at 1 hr), cadmium is unspecifically bound in the cytoplasm and has entered the nucleus *in vivo* (Table IA, Exp 3).

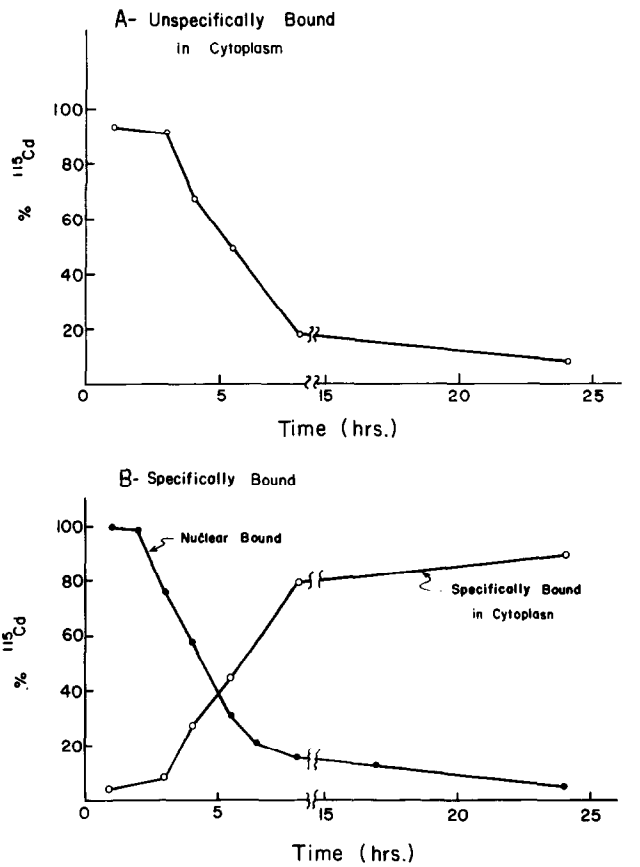

Fig. 3. Intracellular Cd profile. The cytoplasmic Cd-profiles were compiled by totalling the counts of the unspecifically and specifically bound peaks as identified in Fig. 1 and 2 [on the average the sum of the two peaks represents 96.2% of the counts in the total column eluate]. Individual points reflect: total counts in peak/total counts in profile. The % nuclear bound Cd is derived from the nuclear to cytoplasmic ratio of cpm/mg DNA: cpm/mg protein.

TABLE I. Cadmium Uptake in Nuclei

Incubation Treatment	^{115}Cadmium Incubation Time (Hrs)		^{115}Cadmium Nuclei Uptake cpm/mg DNA		
			Undialyzed	Dialyzed	
	in vivo	in vitro[a]		SSC/100	EDTA-SSC/100
A. UNFRACTIONATED:[d]					
Total homogenate treated with ^{115}cadmium					
Experiment 1[c]	0.0	0.5[b]	7955 ± 14		
Experiment 2[c]	0.0	0.5	6340 ± 7	6222 ± 4	1478 ± 15
Experiment 3	1.0	0.0	8658 ± 14	8286 ± 14	1927 ± 25
Experiment 4	20.0	0.0	1215 ± 9	1200 ± 12	649 ± 13
B. FRACTIONATED:					
Nuclei - Cytoplasmic					
(I) Control-Exptl	20.0	1.0	40		
	24.0	1.0	37		
(II) Exptl-Control	20.0	1.0	1087		
	24.0	1.0	904		
(III) Exptl-Exptl	20.0	1.0	1192		

[a] Unless specified, samples were incubated at 0-4° C.
[b] Samples were incubated at 37° C.
[c] Samples were incubated with 2 μCi ^{115}Cd/μmole of Cd for times indicated, prior to nuclei isolation.
[d] All cpm/mg DNA represent the mean of three experiments ± % average deviation from the mean.

The same phenomenon is observed *in vitro* when cadmium is added to control total homogenate and incubated at two different temperatures (Table IA, Experiments 1 and 2). In contrast, at twenty and twenty four hours, when most of the cadmium is bound to CdBP and control nuclei are incubated with 20-24 hour cytoplasmic fraction (Table IB, (I)) there is essentially no uptake of metal by the nuclei. Likewise, when the twenty or twenty four hour nuclear fractions are incubated with the control cytoplasmic fraction, more than 90% of the cadmium remains with the nuclear fraction (Table IB (II)). Thus the data indicate that in the presence of CdBP cadmium cannot enter the nucleus because it is firmly bound in the cytoplasm, however, in the absence of CdBP the cadmium remains in the nucleus suggesting that unspecific cytoplasmic cadmium binding proteins alone are not responsible for removing cadmium.

In order to determine how tightly cadmium is bound in nuclei, dialysis experiments were carried out against SSC/100 (a buffer which solubilizes purified nuclei) in the presence and absence of EDTA (a strong chelating agent for cadmium (16)). Essentially no metal was removed by SSC/100 alone (Table IA, Experiments 2, 3, and 4); upon EDTA-SSC/100 dialysis, 77% and 78% of the nuclear bound cadmium was removed from the *in vitro* 30 minutes nuclei and the *in vivo* 1 hour nuclei, respectively. Yet, only 47% of the metal

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TABLE I. Livers were homogenized as described in text. In A, experiments 1 & 2, 40 ml of total homogenate were incubated with exogenous cadmium; in experiments 3 and 4, the total homogenate was obtained from animals that were treated with cadmium prior to nuclei isolation. Purified nuclei (3-5 mg DNA) from experiments 2, 3 and 4 were dialyzed at room temperature against either 4 liters SSC/100 for 6 hrs or 0.25 M EDTA (0.5 liters) for one hr and SSC/100 (4 liters) for 5 hrs. Same results are obtained by dialyzing against SSC/100 prior to treatment with EDTA-SSC/100. In B the total homogenates, from control (no cadmium) and experimental (10 μci ^{115}Cd/5 μmoles Cd) animals, were separated by centrifugation (700g spin) into cytoplasmic fractions and crude nuclear pellets, each divided into two portions and the supernatants used as a substrate for incubating the nuclear pellets as follows: one half the nuclear pellet from control was placed in one half of experimental supernatant; one portion of the experimental nuclei was placed in control supernatant and the remaining experimental pellet was replaced in experimental supernatant. The reconstituted samples ((I), (II) and (III)) were incubated at 0-4° C for 1 hr with stirring prior to the isolation of nuclei.

was removed from 20 hour purified nuclei (Table IA, Exp. 4). Apparently, the bound metal remaining in the nucleus at 20 hours is more tightly held than the metal at 1 hour since EDTA removes proportionally less at 20 hours.

Other investigators have shown that transcriptional inhibitors are partially effective in preventing the synthesis of Cd-binding protein when administered prior to metal challenge yet have little or no effect when given three hours after metal treatment (8, 9). By focusing on the intracellular distribution of metal during the early period after challenge, we show that high levels of nuclear cadmium also are correlated with this lag period (2-3 hours) prior to production of significant amounts of CdBP. However, this so called lag period is an artifact of the system (Bryan & Hidalgo, unpublished observation) due to limited sensitivity of the methods now used for detection of low concentrations of CdBP; the lag exists only because the levels of CdBP are insufficient to remove measurable amounts of cadmium from the nucleus. In the presence of carrier free ^{115}cadmium, the apparent time required for induction of CdBP is less than an hour. It appears that no measurable time is required for the movement of cadmium from the cytoplasm into the nucleus, prior to CdBP synthesis, both in vivo and in vitro. Cadmium seems to enter the nucleus by diffusion and to establish an equilibrium that is affected only by the amount of unspecifically bound metal in the cytoplasm.

Our data also indicate: (1) the uptake and disappearance of cadmium from the nucleus is well correlated with the appearance of CdBP in the cytoplasm and (2) cadmium enters the nucleus and binds to nuclear material in such a manner that none of it is removed after solubilizing the material and dialyzing it against a buffer of low ionic strength. A fraction of the metal cannot be removed even by a chelating agent under mild conditions.

Although the role of heavy metals in the nucleus has been thus far largely ignored, their presence in this cellular organelle has been established (14, 15, 19). Copper and mercury appear to be selectively bound

to chromatin constituents (19). It is possible that nuclear bound metals are not biologically inert. The fact that cadmium is in the nucleus (at maximum concentrations) during CdBP-mRNA synthesis (8) suggests that the metal could be acting as an effector for CdBP production. We have shown that the metal is removed from the nucleus as CdBP cytoplasmic levels increase. This may provide an effective feedback mechanism for regulating CdBP synthesis: as the level of nuclear cadmium drops, so does the synthesis of CdBP-mRNA. Further studies of the intracellular movement of cadmium and other metals might be useful in gaining insight into eukaryotic regulatory mechanisms.

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REFERENCES

1. Wisniewska, J. M., Trojanowska, B., Piotrowski, J. and Jakubowski, M. (1970) Tox. Applied Pharm. 16, 754-763.
2. Evans, G. W. (1973) Phys. Rev. 53, 535-569.
3. Bremner, I., and Davies, N. T. (1973) Trace Metal Elements in Animal Metabolism pp. 493-496. University Park Press, Baltimore.
4. Bremner, I. and Davies, N. T. (1975) Biochem. J. 149, 733-738.
5. Richards, P. M., and Cousins, R. J. (1975) Biochem and Biophys. Research Comm. 64, No. 4, 1215-1223.
6. Schroeder, H. A., (1974) The Poisons Around Us, pp. 92, Indiana University Press, Bloomington.
7. Colucci, A. V., Winge, D., and Krasno, J. (1975) Arch. Environ. Health 30 153-157.
8. Squibb, K., and Cousins, R. J. (1974) Physiol. Biochem. 4, 24-30.
9. Cousins, R. J. (1973) Trace Metal Elements in Animal Metabolism, pp. 503-505, University Park Press, Baltimore.
10. Schacterle, G. R., and Pollack, R. L. (1973) Anal. Biochem. 51, 654-655.
11. Kagi, J. R. H., and Vallee, B. L. (1960) J. Biol. Chem. 235, 3460-3465.
12. Kagi, J. R. H., Himnelhoch, S. R., Whanger, P. O., Bethune, J. L, and Vallee, B. L. (1974) J. Biol. Chem. 249, 3537-3542.
13. Winge, D., Krasno, J., and Colucci, A. V. (1973) Trace Metal Elements in Animal Metabolism pp. 500-502, University Park Press, Baltimore.
14. Bryan, S. E., Guy, A. L., Hardy, K. J. (1974) Biochemistry 13, 313-319.
15. Bryan, S. E., Lambert, C., Hardy, K. J., and Simons, S. (1974) Science 29, 186-187.
16. Davidson, E. H., and Britten, R. V. (1973) Quart. Rev. Biol. 565-613.
17. Britten, R. J., and Davidson, E. H. (1969) Science, 165, 349-357.
18. Steggles, A. W., Spelsberg, T. C., and O'Malley, B. W. (1971) Biochem. Biophys. Res. Comm. 43, 20-27.
19. Bryan, S. E., Simons, S. J., Vizard, D. L., and Hardy, K. J. (1975) In preparation.