9. _____, *Diabetes* **29** (Suppl. 2), 25A (1980).
10. E. Kohen *et al.*, *Int. J. Bio-Med. Computing* **11**, 305 (1980).
11. R. Michaels, *J. Histochem. Cytochem.* **29**, 901 (Abstr.) (1981); *ibid.*, in press; for paraffin-embedded tissue, see T. Reaves and J. Hayward, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6009 (1979).
12. The proteolytic digestion of pancreatic tissue during islet isolation may alter the islet cell gap junctions [M. Shimo and F. Clementi, *J. Ultrastruct. Res.* **59**, 101 (1977)]. To avoid perturbing the cells, especially those located at the periphery, we used minced pancreas digested with only 1 mg of Sigma type V collagenase per milliliter of pancreas. Islets were then gently hand-picked from the surrounding acinar tissue. This isolation procedure does not appear to alter the peripheral cell coupling because the injected cells that were exposed on the islet surface and those protected by several layers of cells exhibited the same patterns of coupling.
13. P. Lacy and M. Kostianovsky, *Diabetes* **16**, 35 (1967).
14. E. Yavin and C. Yavin, *J. Cell Biol.* **62**, 540 (1974).
15. Glass micropipettes were filled with a 4 percent aqueous solution of Lucifer Yellow CH (W. Stewart, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Md.). The dye was passed from the electrode with negative current pulses (7 to 9 nA). When it was questionable whether the electrode had penetrated only one cell during the injection (as determined by problems during the injection or from photographs taken prior to resin removal indicating more than one highly fluorescent cell), the islet was discarded.
16. W. Stewart, *Cell* **14**, 741 (1978).
17. B. Lane and D. Europa, *J. Histochem. Cytochem.* **13**, 579 (1965).
18. Two types of controls were carried out. In the first, normal serum was substituted for the primary antibody. In the second, antibodies were preabsorbed with their corresponding antigen (100 μg/ml) prior to application to sections. In both cases, controls were negative with no specific binding of the secondary antibody to the sections.
19. Primary antibodies were kindly donated by G. Bauer (guinea pig antibody to insulin), S. Erlandsen (guinea pig antibody to glucagon), R. Elde (rabbit antibody to somatostatin), and R. Chance (rabbit antibody to pancreatic polypeptide).
20. A. Coons, *Int. Rev. Cytol.* **5**, 1 (1956).
21. D. Baetens, F. Malaisse-Lagae, A. Perrelet, L. Orci, *Science* **206**, 1323 (1979).
22. There are limitations in attempting to quantitate the number of dye-containing cells in fixed and embedded tissue. Background fluorescence from the embedding resin (*16*), fading of the dye, or low levels of dye molecules in some of the cells may prevent detection of all of the coupled cells. Therefore, negative results (that is, the lack of visible dye transfer) from either a group of cells or from an individual injected cell need to be interpreted carefully. We report here only positive dye-coupling results, although it should be noted that we were unable to detect any dye transfer from four additional injected cells during the 5-minute injection periods. We believe it is possible to make fairly accurate comparisons of the effects of incubating conditions on the dye-coupling capabilities of the cells in this system if the preservation of the tissue is the same in all cases. To avoid confusion, photographs were taken immediately after islet injection, prior to resin removal, and after resin removal and immunocytochemical staining for comparison. Throughout the procedure, Lucifer Yellow remained in the tissue. In all instances the number of cells that appeared to contain dye prior to resin removal was the same as that after resin removal, and these values corresponded to the number of cells that appeared to be involved in the transfer during the dye injection.
23. P. Meda, J. Denef, A. Perrelet, L. Orci, *Am. J. Physiol.* **238**, C114 (1980).
24. P. Meda, A. Perrelet, L. Orci, *J. Cell Biol.* **82**, 441 (1979).
25. P. Meda, P. Halban, A. Perrelet, A. E. Renold, L. Orci, *Science* **209**, 1026 (1980).
26. R. H. Unger, A. Eisentraut, M. McCall, L. Madison, *J. Clin. Invest.* **41**, 682 (1962).
27. J. Vance, K. Buchanan, D. Challoner, R. Williams, *Diabetes* **17**, 187 (1968).
28. This work was supported by a grant from the American Diabetes Association of Minnesota, No. 79–60. We thank W. Stewart for the Lucifer Yellow CH and D. Spray for carefully reviewing the manuscript.

23 June 1981; revised 28 August 1981

Further Heterogeneity of Human α Interferon mRNA Species

Abstract. *Translationally active (in* Xenopus *oocytes) human α interferon (IFN) messenger RNA's (mRNA's) derived from Sendai virus–induced leukocyte cultures display a bimodal distribution of RNA lengths on electrophoresis through agarose-CH_3HgOH gels. The major population (α_S) consists of mRNA of length 0.7 to 1.4 kilobases, while the minor population (α_L) consists of RNA of length 1.6 to 3.5 kilobases. Induction of human leukocytes in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; 100 micromolar) appears to inhibit the accumulation of IFN-α_S and to enhance that of IFN-α_L mRNA's (average length about 1.8 kilobases in preparations from DRB-treated cells). Interferons derived from the α_S mRNA's represent the group of previously recognized α interferons while the α_L interferons are distinguishable from this group by their lower heterospecific activity on bovine cells compared to human cells, their apparent slower mobility in sodium dodecyl sulfate–polyacrylamide gels, and their apparent heteroclitic response toward an antiserum to IFN-α.*

Recent advances in the molecular cloning of human interferons (IFN's) and their complementary DNA (cDNA) and chromosomal DNA sequences have provided detailed information about the structure of some human interferon α and β genes (*1–3*). Several investigators (*2*) have cloned and characterized at least eight distinct cross-hybridizing species of cDNA derived from sucrose-gradient enriched 12*S* interferon messenger RNA (mRNA) obtained from either virus-induced human leukocyte or virus-induced human lymphoblastoid cell cultures and have also characterized the structure of interferon genes which correspond to these species. Briefly, these genes do not have introns and appear to correspond to mature polyadenylated mRNA's approximately 0.8 to 1.4 kb in length (collectively referred to by us as IFN-α_S mRNA's). In our present study, we used RNA electrophoresis under stringent denaturing conditions through

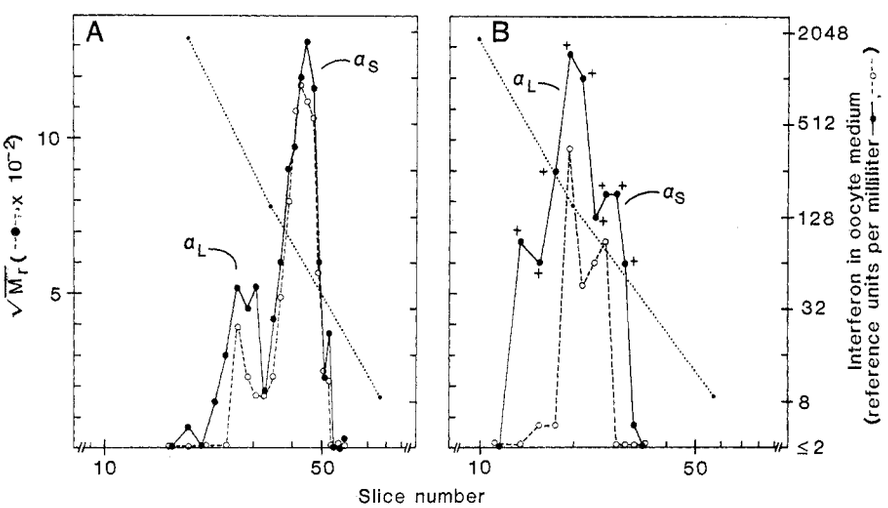

Fig. 1. Agarose-CH_3HgOH gel electrophoresis of IFN-α mRNA's induced by Sendai virus in human leukocyte (peripheral blood buffy coat) cultures in the absence (A) or presence (B) of DRB. Polyadenylated RNA (*11*) was extracted from interferon-primed (100 reference units per milliliter; 2 hours at 37°C) Sendai virus–induced (150 hemagglutination units per milliliter) human leukocytes (~ 5×10^6 cells per milliliter) (*5*) at various times up to 10 hours after the beginning of induction and was pooled. In (B), DRB (Calbiochem; 100 μ*M*) was present from 1 to 2 minutes prior to the addition of Sendai virus until the time of cell harvest. The RNA [23 μg derived from 2.5×10^9 cells in panel (A) and 15 μg derived from 1.6×10^9 cells in (B)] was mixed with ^{32}P-labeled cytoplasmic RNA from HeLa cells; it was denatured in 10 m*M* CH_3HgOH and was then subjected to electrophoresis through a 1.5 percent (A) or 2 percent (B) agarose–10 m*M* CH_3HgOH tube gel (11 by 0.6 cm) (*11*). Adjacent 1-mm gel slices were pooled in pairs; the RNA was eluted and dissolved in 10 μl of sterile, distilled water; and 1 μl of each sample was given by microinjection to groups of *Xenopus* oocytes (NASCO) incubated in 0.2 ml of Barth's medium (*11*). Approximately 24 hours later the interferon content of the oocyte incubation medium was assayed on human cells (GM 2504 or GM 2767, ●) or bovine cells (EBTr, ○) in a semimicroassay of cytopathic effect inhibition; vesicular stomatitis virus was the challenge virus and the result is expressed in terms of the 69/19 or G-023-901-527 NIH reference standards for human interferon. The RNA length was calibrated with marker 28*S*, 18*S*, and 4*S* RNA (dotted line). The interferon activity on human cells was completely neutralized by antiserum to IFN-α (*12*) but was unaffected by antiserum to IFN-β_1 (*13*) (indicated by plus symbols).

0036-8075/81/1113-0803S01.00/0

agarose-CH_3HgOH gels to demonstrate the existence in leukocyte-derived RNA of a second heterodisperse population of human IFN-α mRNA species of length 1.6 to 3.5 kb which code for α interferons that are distinct from the previously described α_s interferons. The inclusion of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (100 μ*M*) (*4*) in the leukocyte induction procedure (*5*) leads to an enhanced accumulation of IFN-α_L mRNA as well as to the enhanced production of an unusual set of human interferons—the α_L interferons. These observations suggest that the system of human α interferons is more complex than has been generally appreciated.

We show the electrophoretic profile of interferon mRNA derived from interferon-primed, Sendai virus–induced human leukocytes (peripheral blood buffy coat cells) maintained in culture in the absence (Fig. 1A) or the presence of DRB (Fig. 1B). Figure 1A shows that the major population of human leukocyte IFN-α mRNA ("IFN-α_s") is between 0.7 and 1.4 kb (average length ~ 1 kb) in length, that there is a second population of longer IFN-α mRNA (> 1.4 kb, "IFN-α_L"), and that the ratio of interferon activity of the oocyte translation products on human and bovine cells is approximately equal for the α_s mRNA population whereas the translation products of IFN-α_L mRNA's are less active on bovine cells than on human cells. The major α_s mRNA peak corresponds to RNA sedimenting at approximately 13*S* on a sucrose gradient (*2*). Furthermore, the approximate equivalence of IFN-α activity on human and bovine cells of the IFN-α_s mRNA translation products is in agreement with the approximate equivalence of human:bovine activity of previously reported human leukocyte interferon preparations (*6*). The inclusion of DRB during the induction inhibits the accumulation of IFN-α_s mRNA's, but markedly enhances that of a population of long IFN mRNA species (collectively referred to as IFN-α_L mRNA's) with an average length of ~ 1.8 kb (Fig. 1B). The oocyte translation products derived from α_L mRNA species display lower activity on bovine than on human cells and are completely neutralized by antiserum to IFN-α but not by antiserum to IFN-β (data not shown).

Human α interferons produced by leukocytes in the presence of DRB are distinguishable from those produced in its absence (Fig. 2). Interferons produced in the absence of DRB represent the well-known Cantell interferons (*6*) (Fig. 2A) which (i) display a biomodal protein distribution after electrophoresis through sodium dodecyl sulfate (SDS)–polyacrylamide gels with most of the interferon activity in a population of molecules distributed around 18,000 and some around 22,000; (ii) the high molecular weight population has approximately one-half to one-third as much activity on bovine cells as on human cells, whereas the low molecular weight population is far more active on bovine cells than on human cells; and (iii) the material in the leading edge of the low molecular weight population is exceptionally active on bovine cells with very little activity on human cells. In contrast, leukocyte interferons produced as a group in the presence of DRB (i) are proteins with a detectably slower electrophoretic mobility and (ii) are less active on bovine cells than on human cells. The lower activity on bovine cells of interferons produced in the presence of DRB is consistent with observations described in Fig. 1B. Quantitative serologic tests reveal a further difference between the α_s (Fig. 2A) and α_L (Fig. 2B) interferons. The neutralization titer (*7*) of rabbit antiserum to IFN-α against interferons produced in the absence of DRB was in the range of 200 to 400 neutralization units per milliliter (Fig. 2A). The neutralization titers of the same preparation of antiserum against several of the interferon species induced in the presence of DRB were in the range 2000 to 6000 neutralization units per milliliter (Fig. 2B).

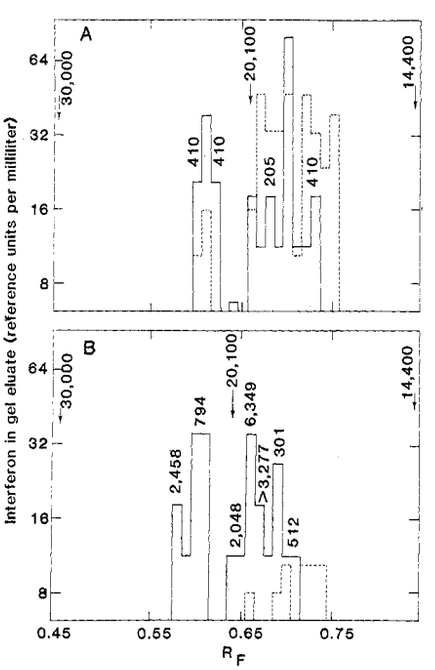

Fig. 2. Characterization of α interferons produced by human leukocytes in the absence (A) or presence (B) of DRB. IFN-α proteins produced by 4 hours after induction in the cultures described in Fig. 1 (100 μl of medium) were subjected to electrophoresis through SDS–10 percent polyacrylamide gels (*14*). After the dye front had migrated approximately 20 cm, the gel was sliced into 2-mm segments, and the interferons were eluted and assayed on human (solid line) or bovine (broken line) cells. Interferons from selected gel slices were tested for their ability to be neutralized by a rabbit antiserum to IFN-α (produced at Meloy Laboratories against IFN-α derived from human leukocytes). The neutralization titers (*7*) are indicated above the respective gel segments. The molecular weight markers are carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Differences in the heterospecific activity and in the neutralization titers similar to those described in Fig. 2 are also observed when crude interferons produced by the Cantell (*5*) procedure (mainly α_s) are compared to those produced by the DRB-modified procedure (mainly α_L) (*8*). Furthermore, long (~ 1.8 kb) polyadenylated IFN-α mRNA species have also been observed in the cytoplasmic compartment in Sendai virus–induced human lymphoblastoid (Namalva) cells (*9, 10*).

Recent experiments indicate that there is no detectable cross-hybridization between IFN-α_L mRNA's and a ^{32}P-labeled IFN-α_s–specific DNA probe even when the hybridization is carried out under relaxed conditions.

PRAVINKUMAR B. SEHGAL
ANURAG D. SAGAR
Rockefeller University, New York 10021
IRWIN A. BRAUDE
Meloy Laboratories, Inc., Springfield, Virginia 22151

References and Notes

1. Human interferons are classified into α ("leukocyte"), β ("fibroblast"), and γ ("immune") interferon on the basis of their antigenic properties [IFN Nomenclature Committee, *Nature (London)* **286**, 110 (1980)]. Thus antiserums to IFN-α neutralize only α-IFN's but not β-IFN's and so forth.
2. S. Nagata *et al.*, *Nature (London)* **284**, 316 (1980); S. Nagata, N. Mantei, C. Weissmann *ibid.* **287**, 401 (1980); M. Streuli, S. Nagata, C. Weissmann, *Science* **209**, 1343 (1980); S. Nagata, C. Brack, K. Henco, A. Schambock, C. Weissmann, *J. Interferon Res.* **1**, 333 (1981); D. V. Goeddel *et al.*, *Nature (London)* **287**, 411 (1980); D. V. Goeddel *et al.*, *ibid.* **290**, 20 (1981).
3. T. Taniguchi, M. Sakai, Y. Fujii-Kuriyama, M. Muramatsu, S. Kobayashi, T. Sudo, *Proc. Jpn. Acad.* **55** (Ser. B), 464 (1979); T. Taniguchi, S. Ohno, Y. Fujii-Kuriyama, M. Muramatsu, *Gene* **10**, 11 (1980); T. Taniguchi, L. Guarente, T. M. Roberts, D. Kimelman, J. Douchan III, M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5230 (1980); R. Derynck *et al.*, *Nature (London)* **287**, 193 (1980); R. Derynck *et al.*, *ibid.* **285**, 542 (1980); D. V. Goeddel *et al.*, *Nucleic Acids Res.* **8**, 4057 (1980).
4. I. Tamm, R. Hand, L. A. Caliquiri, *J. Cell Biol.* **69**, 229 (1976); P. B. Sehgal, J. E. Darnell, I. Tamm, *Cell* **9**, 473 (1976); P. B. Sehgal and I. Tamm, *Antibiot. Chemother. Basel* **27**, 93 (1980).
5. K. Cantell and S. Hirvonen, *Tex. Rep. Biol. Med.* **35**, 138 (1977); _____, H. L. Kauppinen, G. Myllyla, *Methods Enzymol.*, in press.
6. I. Gresser, M. T. Bandu, D. Brouty-Boye, M. Tovey, *Nature (London)* **251**, 543 (1974); W. E. Stewart II and J. Desmyter, *Virology* **67**, 68 (1975); I. A. Braude, L. S. Lin, W. E. Stewart II, *Biochem Biophys. Res. Commun.* **89**, 612 (1979); W. E. Stewart II, F. H. Sarkar, H. Taira, A. Hall, S. Nagata, C. Weissmann, *Gene* **11**, 181 (1980).

7. The neutralization titer per milliliter is the product of the reciprocal of the highest dilution of the antiserum which completely neutralizes a sample of interferon and the titer of that sample of interferon expressed in reference units per milliliter.
8. I. A. Braude, C. Tarr, A. D. Sagar, P. B. Sehgal, in preparation.
9. S. L. Berger, M. J. M. Hitchcock, K. C. Zoon, C. S. Birkenmeier, R. M. Friedman, E. H. Chang, *J. Biol. Chem.* **255**, 2955 (1980).
10. A. D. Sagar *et al.*, *Nucleic Acids Res.* **9**, 149 (1981).
11. P. B. Sehgal and A. D. Sagar, *Nature (London)* **287**, 95 (1980).
12. Provided by W. E. Stewart II, originally prepared by Dr. K. Fantes.
13. Provided by Y. H. Tan.
14. I. A. Braude, L. S. Lin, W. E. Stewart II, *Biochem. Biophys. Res. Commun.* **89**, 612 (1979).
15. We thank Dr. I. Tamm for discussions and I. Augenzucker and R. Biehl for technical assistance, Dr. G. C. Tarr for help with the IFN-α production, and Drs. Y. H. Tan and W. E. Stewart II for providing us some of the antiserums to interferon used. Supported in part by grant NIAID AI-16262 (to P.B.S.), by a predoctoral institutional training grant from the National Institutes of Health (to A.D.S.), and by a junior faculty research award from the American Cancer Society (P.B.S.).

6 April 1981; revised 5 August 1981

Detection of Circulating Metallothionein in Rats Injected with Zinc or Cadmium

Abstract. *Circulating metallothionein was measured by radioimmunoassay over a 13-day period in male Sprague-Dawley rats that received a sequence of three intraperitoneal injections (at 3-day intervals) of either 5 milligrams of zinc or 0.8 milligrams of cadmium per kilogram of body weight. These amounts of zinc and cadmium produced metallothionein concentrations in the range of 2 to 5 nanograms per milliliter of serum (zinc) and 2 to 15 nanograms per milliliter of serum (cadmium). In control rats given saline injections over the same period the metallothionein concentration ranged from 1 to 3 nanograms per milliliter of serum.*

Numerous investigators, using physicochemical methods, have detected metallothionein (Mt) in rat and mouse plasma (*1–3*). However, the accurate measurement of low concentrations of Mt or its precursor thionein in the serum of animals exposed to metals (for example, Zn, Cd, Cu, Hg) for short periods has not been achieved to date because of the lack of a sensitive measurement technique (*4*). The recent development of a radioimmunoassay (RIA) for Mt with a detection limit of less than 100 pg now permits accurate measurements of circulating Mt (*5*). We used this method to measure Mt in the serum of rats given intraperitoneal injections of zinc and cadmium.

Male Sprague-Dawley rats (275 to 325 g) received three injections (spaced 3 days apart) of either $ZnSO_4$ in saline (5 mg of zinc per kilogram of body weight) or $CdCl_2$ in saline (0.8 mg of cadmium per kilogram of body weight); controls received saline only. The protocol was established by conducting dose-response experiments (*6*) and comparing our findings with those of others (*7, 8*). Blood, approximately 1.5 ml per sample, was obtained retro-orbitally at intervals of 18 or 24 hours (with a few instances of 6- or 12-hour intervals). The samples were allowed to clot, the serum was removed and centrifuged at 600*g* for 10 minutes to completely remove the cells, and the samples were frozen. Subsequently, four 50-μl portions from each sample were radioimmunoassayed for Mt.

Serum concentrations of Mt were determined by the use of simultaneously prepared logit-log regressions (*9*) derived from standardizing data relating the concentration of unlabeled Mt to the percentage of bound protein (the ^{125}I-labeled Mt used in the RIA). Significance levels of differences of means were evaluated on the assumption of a two-tailed Student *t* distribution and populations of unequal variances (*10*).

The curves (Fig. 1) relating Mt concentration to time after the initial injection are based on the values associated with blood samples being obtained at intervals of 18 or 24 hours; a few 12-hour values are included (*11*). The mean concentrations for the controls in each injection period are not statistically distinguishable; this encourages the belief that the injection and bleeding protocols did not induce Mt production independent of metal injection. The fiducial limits at 99 percent confidence (0.60 to 2.98 ng of Mt per milliliter) of the mean control concentration over the 13-day period are in accord with values previously found for Mt concentrations in the serum of humans with no history of significant exposure to heavy metals (*12*).

The nature of the response to the multiple injections of zinc (sequential relative maxima showing an increase with each injection) was characteristic of responses to multiple injections of zinc at nontoxic levels in the preliminary dose-response experiments (*6*). This behavior accords with results obtained by others (*13, 14*). The return of Mt concentration to control levels in about 4 days after the last injection is in accord with the reported short biological half-life of zinc and of the Mt it induces (*13, 15*).

In contrast to the slight initial response to the 5-mg/kg dose of zinc, the response to 0.8 mg of cadmium per kilogram led to a rapid increase in Mt concentrations (about 7 ng/ml per day) during the first 12 hours after injection (*16*), this peaking at about 48 hours and gradually subsiding with brief interruptions after the second and third injections. The response remained relatively constant at about four times the control levels in the terminal period (9 to 13 days); this response appears to be in accord with the reported long biological half-life of cadmium (*17, 18*).

The difference in character of the response to nontoxic doses of zinc and of cadmium in a sequence of multiple injections (a succession of increasing relative maxima followed by a return to control levels contrasted to an initial maximum followed by a gradual decrease to a level about four times control concentrations) is certainly in part dose-related (*6, 16*), but it must also reflect a difference between the two metals in the combined processes of initial Mt induction and the subsequent kinetics of metal retention, recirculation, and repeated Mt induction.

From our results on induced Mt concentrations in serum and the results of others on cadmium and zinc concentrations in plasma or serum after intraperitoneal or subcutaneous injections of these metals (*18–21*), we may arrive at some conclusions regarding Mt in its role as a metal-binding protein. Typical measurements at 1 to 30 minutes after the injection of cadmium indicate plasma concentrations about one-fifth of the injected whole-body concentration, these decreasing rapidly during the first few hours, becoming about 10^{-4} of the injected dose after 24 to 48 hours, and remaining essentially constant for the next few days. On this basis, our initial (total) injection of about 250 μg per rat would have led in minutes to the appearance of about 50 μg (total) of cadmium in serum, this decreasing to about 25 ng (total) at 24 to 48 hours after injection. The observed increase in Mt concentration after the initial injection implies a maximum Mt concentration in the first few hours of less than 1 ng/ml or less than 20 ng (total) in serum. Even if saturated with cadmium this amount of Mt could bind only about 2 ng of cadmium and could not be a major carrier of serum cadmium in this period. The situation is even more extreme for zinc. The results are thus in accord with experiments which indicate

0036-8075/81/1113-0805$01.00/0