METAL ION HOMEOSTASIS: MOLECULAR BIOLOGY AND CHEMISTRY

Organizers: Dennis Winge and Dean Hamer
April 10-16, 1988

Page

Plenary Sessions

April 11:
Zinc Fingers in DNA Binding Proteins..322
Metallothionein Gene Cluster and Regulation by Metals....................................324

April 12:
Role of Iron in the Regulation of Iron Transport and Storage Proteins - I...............325
Structural Aspects of Transport/Storage Proteins..326

April 13:
Role of Iron in the Regulation of Iron Transport and Storage Proteins - II..............327
Chemistry and Biology of Metalloregulatory Switches..328

April 14:
Metallothioneins...330
Banquet Address..331

April 15:
Metallothionein and Metallothionein-like Peptides..332
Physiology and Diseases of Unbalanced Metal Homeostasis................................333
Phytochelatins in Plants..334

Poster Sessions

April 11:
Gene Regulation (Z 100-123)..336

April 12:
Structure and Chemistry of Metalloproteins (Z 200-216)...................................343

April 13:
Detoxification and Disease States (Z 300-313)...350

April 14:
Metal Metabolism and Utilization (Z 400-411)...354

Metal Ion Homeostasis: Molecular Biology and Chemistry

Zinc Fingers in DNA Binding Proteins

Z 001 METAL-BINDING DOMAINS, Jeremy M. Berg, Department of Chemistry, The Johns Hopkins University, Baltimore MD 21218

Metal-binding domains are comprised of a metal ion (or metal ions) that has its coordination sites saturated by ligands which come from a short stretch of amino acid sequence. Recently, it has been discovered that several classes of proteins involved in nucleic acid binding and gene regulation may contain metal-binding domains. Two approaches to studying these systems will be discussed. The first involves analysis of amino acid sequences and includes methods for identifying potential metal-binding domain forming sequences and attempts to predict three-dimensional structures of metal-binding domains based on the structures of other metalloproteins and of small inorganic complexes. Using these methods an attractive model for the "zinc finger" metal-binding domains from Transcription Factor IIIA and related proteins has been developed. The second approach involves the preparation of peptides which correspond to individual metal-binding domains. The peptides can be characterized with regard to their metal-binding and structural properties using a variety of chemical and spectroscopic techniques. This approach has been applied to the "zinc finger" domains among other systems.

Z 002 *TAT*-III PROTEIN FROM HIV FORMS A METAL-LINKED DIMER, Alan D. Frankel, David S. Bredt and Carl O. Pabo, Department of Molecular Biology & Genetics and the Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Tat-III, the transactivating protein from human immunodeficiency virus (HIV), forms a metal-linked dimer, with Zn^{2+} or Cd^{2+} ions bridging cysteine-rich regions from each monomer. Ultraviolet absorption spectra show that *tat*-III binds two Zn^{2+} or two Cd^{2+} ions per monomer, and electrophoresis of the *tat*-III-metal complexes on native and SDS gels demonstrates that the protein forms metal-linked dimers. Partial proteolysis and circular dichroism spectra suggest that metal binding does not mediate a global folding transition for *tat*-III. A synthetic peptide containing just the cysteine-rich region of *tat*-III also forms metal-linked dimers and can form heterodimers with the intact protein (thus blocking formation of the normal *tat*-III dimer). Metal-linked dimerization could play an important role in transactivation, and our results may have important implications for drug design and AIDS therapies.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 003 ZINC DOMAINS IN NONSPECIFIC NUCLEIC ACID BINDING PROTEINS, D.P. Giedroc and J.E. Coleman, Dept. of Molecular Biophysics & Biochemistry, Yale Univ. School of Medicine, New Haven, CT 06510. Gene 32 protein from bacteriophage T4 binds stoichiometrically and cooperatively to single-stranded DNA without sequence specificity. The protein contains 1 g at Zn(II)/mol which can be exchanged with Cd(II) or Co(II) (1). The metal ion greatly enhances cooperative binding to an "infinite" length ssDNA lattice with little effect on noncooperative binding to a single binding site oligodeoxy-ribonucleotide (2). NMR of ^{113}Cd(II)-substituted g32P shows a single resonance 638 ppm downfield from aqueous ^{113}Cd(II) with no evidence for anion (solvent) access to the inner metal sphere; all data are consistent with tetrahedral NS_3 coordination. Spectroscopic characterization of g32P metal site mutants (under construction) will further support the assignment of Cys^{77}, His^{81}, Cys^{87} and Cys^{90} as providing metal ligand donor atoms as previously proposed (1). Noncooperatively [d(pA)$_6$] or cooperatively [poly(dT)] binding DNA ligand causes little if any perturbation in the Co(II) g32P visible absorption spectrum or the ^{113}Cd(II) g32P chemical shift ruling out metal site expansion, ligand exchange or large conformational changes transmitted through the metal ion chelate as a result of DNA binding. Differential scanning calorimetry and limited proteolysis experiments show that the metal ion stabilizes the folded protein (1-3). Cooperative binding to poly(dT) results in a further stabilization of all metallo g32Ps which are uniformly characterized by sharp asymmetric "cooperative" denaturational transitions; in contrast, apo g32P exhibits insignificant stabilization upon stoichiometric binding to poly(dT) and a denaturational transition consistent with a simple two-step process (3). Further examination of the thermodynamics of g32P unfolding support the contention that a negative change in the entropy of the folded apo protein occurs as a result of Zn(II) binding as observed zinc-ligand bond enthalpies are not fully realized in the relative T_m and free energy values of apo and metallo g32Ps. Consistent with this, far UV CD difference spectroscopy shows that the Zn(II) binding to apo g32P induces significant negative ellipticity at 222 nm (~1.5 helical turns). Cd(II) binding to the apo protein results in the same degree of induced optical activity which eliminates potentially significant metal sulfur charge transfer transitions as the source of the induced ellipticity. Such changes in structure seem primarily associated with increasing the magnitude of g32P monomer-monomer interactions which occur on cooperative binding to ssDNA. 14-amino acid Cys_3/His domains are found in the C-terminal processed product of retroviral *gag*-gene polyproteins which are thought to function in nonspecific RNA binding in packing the virion RNA into the capsid structure. To address whether such domains specify zinc binding sites, we have constructed plasmids which direct the expression of the p15 *gag*-gene protein from HIV under control of the P_L promoter in *E. coli*; purification and characterization experiments are in progress.

(1) Giedroc *et al.* (1986) *PNAS 83*, 8452
(2) Giedroc *et al.* (1987) *Biochemistry 26*, 5251
(3) Keating *et al.* (1988) *Biochemistry*, in press.

Z 004 "FINGERPRINTING" THE TFIIIA-5S GENE COMPLEX, Thomas D. Tullius, Mair E. A. Churchill and Jeffrey Hayes, Department of Chemistry, The Johns Hopkins University, Baltimore MD 21218.

Transcription factor IIIA (TFIIIA), perhaps the best characterized eukaryotic gene regulatory protein, has been proposed to bind to DNA through structural domains called "zinc fingers." Evidence for this proposal has come from analysis of the zinc content of the protein, and from recognition of the presence of nine imperfect repeats in the sequence of TFIIIA, each of which contains two cysteines and two histidines, which are commonly found as ligands to zinc in metalloproteins. The question remains as to how these nine zinc fingers actually bind to DNA. We have studied directly the structure of the complex of TFIIIA with DNA by use of a new method developed in our laboratory, hydroxyl radical footprinting. This technique makes use of the Fenton reaction of iron(II) with hydrogen peroxide to generate the hydroxyl radical (•OH), which breaks the sugar–phosphate chain of DNA. Bound protein protects DNA from attack by the hydroxyl radical. The small size of the hydroxyl radical leads to very high resolution "footprints" of proteins bound to DNA. We have studied by this method a series of deletion mutants of TFIIIA that lack one or more of the nine zinc fingers. By these experiments we are able to map which of the fingers of TFIIIA interact with which parts of the 50 base pair-long binding site. Based on these data, and a model for the structure of a zinc finger, a detailed picture of the TFIIIA–DNA complex will be presented.

Metallothionein Gene Cluster and Regulation by Metals

Z 005 REGULATION OF METALLOTHIONEIN GENE TRANSCRIPTION. D. Hamer, V. Culotta, P. Furst, R. Hackett, S. Hu and J. Imbert. Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Metal ions such as zinc, copper and cadmium are potent inducers of metallothionein gene transcription, thus providing a homeostatic cellular defense system. We are studying the molecular mechanisms of heavy metal induction both in a simple lower eukaryote, S. cerevisae, and in mammalian cells. Our studies include (1) the construction and characterization of systematic point mutants throughout the metal regulatory DNA sequences; (2) the detection, purification and cloning of proteins that interact with these regions; and (3) the isolation of trans-acting mutants in the DNA-binding factors. Current results suggest that, in both yeast and mammals, metal induction is under complex control mediated by the interactions of specific activator and repressor proteins.

Z 006 TRANSCRIPTION FACTOR SP1 REQUIRES ZINC FOR DNA BINDING AND ACTIVATOR FUNCTIONS, Gunnar Westin and Walter Schaffner, Institut für Molekularbiologie II der Universität Zürich, CH-8093 Zürich, Switzerland.
Zinc is an important cofactor for many enzymes involved in nucleic acid metabolism such as DNA and RNA polymerases, reverse transcriptase and tRNA synthetase. The transcription factors TFIIIA of Xenopus laevis and GAL4 of Saccharomyces cerevisiae also contain zinc ions essential for DNA binding and transcriptional activity. We show that Spl, a promoter-specific transcription factor of vertebrates, requires zinc for binding to its recogniton sequences in vitro and that zinc is also essential for Spl factor-directed transcription. First of all, we show that Spl is involved in transcription directed by the mouse metallothionein-I gene enhancer. In addition, transcription from the SV40 early promoter, which contains multiple Spl binding sites, is metal ion-responsive in HeLa cells. We suggest that Spl is not mainly responsible for metal-induced transcription but rather interacts synergistically with other metal-binding transcription factors to mediate a strong transcriptional response.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Role of Iron in the Regulation of Iron Transport and Storage Proteins - I

Z 007 EUKARYOTIC RNA REGULATORY ELEMENTS: INSIGHTS FROM IRON METABOLISM.
Richard D. Klausner, Matthias W. Hentze, John L. Casey, S. Wright Caughman, David M. Koeller, Tracey A. Rouault and Joe B. Harford, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA.
The uptake and intracellular distribution of iron are highly regulated processes. The two genes that regulate these respective processes encode the transferrin receptor (TfR) and ferritin (Ft). The homeostasis of cellular iron metabolism is assured by the rapid regulation of the biosynthesis of these two proteins in response to fluctuations in cellular iron levels. Increased cellular iron reduces the synthesis of the TfR and raises the rate of biosynthesis of Ft. The regulation of TfR synthesis is a reflection of altered levels of TfR mRNA while the altered biosynthesis of Ft occurs in the absence of changes in Ft mRNA levels. We have isolated the sequence present in the 5' untranslated region (UTR) of Ft that is responsible for the ability of iron to regulate Ft translation. This sequence, which we have termed an iron responsive element (IRE), is present in all known Ft 5' UTR's including human, rodent and amphibia. This element is capable of forming a characteristic stem loop structure. Both a defined structure and specific residues are conserved and required for IRE function. The iron responsive regulation of TfR mRNA levels can be traced to two distinct loci in the TfR gene. One is a transcription element found in the 5' flanking region of the gene. This is responsible for a three fold sensitivity of transcription to iron. A much larger effect is attributable to an element located in the DNA that encodes the 3' UTR of the TfR mRNA. This region of the mRNA is capable of a high degree of secondary structure and is strikingly conserved between chicken and man. Included in this region are five elements that display the IRE consensus structure and sequence. When each of two of these IRE-like elements are synthesized and transferred to the 5' UTR of an indicator gene, iron dependent translational control is observed. In these constructs the iron dependent translational control is qualitatively identical to that seen in Ft. This suggests that the IRE is an RNA regulatory element which produces different effects via different mechanisms depending upon its context within an RNA transcript.

Z 008 REGULATION OF FERRITIN GENE EXPRESSION IN RELATION TO INTRACELLULAR IRON LEVELS
H.N. Munro, N. Aziz, E.A. Leibold, M. Murray, J. Rogers and K. White, USDA Human Nutrition Center on Aging, Boston, MA 02111 and Department of Applied Biological Sciences, M.I.T., Cambridge, MA 02139.
Ferritin stores iron within a shell of 24 protein subunits. In mammals the subunits are of two kinds, heavy H (M_r 21,000) and light L (M_r 19,000), which display 50% amino acid homology. Nevertheless alpha-helical structure is conserved, confirmed by similar exon patterns and sizes in the expressed H and L genes, thus suggesting derivation of the two subunits from an ancestral subunit gene. In addition to single expressed H and L genes, the rat and human genomes contain a number of pseudogenes of each subunit.

Synthesis of the subunits in response to iron is regulated at both transcriptional and translational levels. Translation is rapidly stimulated by iron administration to animals or cells through a mechanism resistant to actinomycin D. The response is due to mobilization of a large cytoplasmic pool of H and L mRNAs stored as inactive mRNP particles which become recruited onto polysomes for translation. Inspection of the 5'UTR of ferritin H and L mRNAs of human, rat, bullfrog and chicken shows a highly conserved 28-nucleotide sequence near the cap of the 5'region. Constructs made from the 5'UTR and 3'UTR of ferritin L-mRNA and the reading frame of CAT and transfected into hepatoma cells responded to iron administration with CAT synthesis, but lost this response when the first 67 nucleotides of the 5'UTR including the conserved 28-nucleotide region was excised. Computer analysis of this segment of the 5'UTRs of various species display a stem-loop structure, the conserved sequence being at the top of the loop. Cytoplasmic extracts from rat tissues and hepatoma cells were shown by an electrophoretic separation procedure to form RNA-protein complexes with ^{32}P-labeled 5'UTR of both ferritin H and L mRNAs. RNAase T1 mapping of the RNA in the complex showed that the 28-nucleotide sequence was protected. Iron administration to rats or cells affected the pattern of RNA-complex formation. We propose that intracellular iron levels regulate ferritin synthesis at the translational level by causing changes in specific protein binding to a conserved sequence in the 5'UTR of the H and L ferritin mRNAs.

The above mechanism allows rapid recruitment of latent ferritin H and L mRNAs in order to accelerate ferritin synthesis. In addition, nuclear run-off assays show that iron administration to rats preferentially stimulates transcription of liver L-mRNA, thus favoring synthesis of L-rich ferritin shells which favor iron storage. Consequently, ferritin protects the cell against excess iron by two mechanisms, a rapid translational recruitment of ferritin mRNAs stored in bulk, and a preferential increase in L-mRNA through transcriptional enhancement.

Structural Aspects of Transport/Storage Proteins

Z 009 MIXED LIGAND CYANO COMPLEX OF FERRIC TRANSFERRIN,

N. Dennis Chasteen* Susan K. Swope*, Daniel C. Harris**, Hans van Willigen# and John McCracken##, *University of New Hampshire, Durham, NH 03824; **Naval Weapons Center, China Lake, CA 93555; #University of Massachusetts at Boston, Boston, MA 02125; ##Biotechnology Resource in Pulsed EPR Spectroscopy, Albert Einstein College of Medicine, Bronx, NY 10461.

Cellular chelators are probably important for the release of iron from transferrin in vivo. Recent studies have shown that transient mixed-ligand complexes of the type Chel-Fe-transferrin are formed during the exchange of iron between transferrin and chelators such as pyrophosphate. Because of their short lifetimes, it has not been possible to study these complexes in detail. However, cyanide forms a stable mixed-ligand complex with the iron(III) in the C-terminal site of transferrin that is amenable to study. The binding of cyanide converts the iron in this site from high to low spin. The results of equilibrium binding and UV-visible, EPR, ENDOR, and ESEEM spectroscopic studies will be reported. The data provide insight into the structure of the adduct and the mechanism of chelate mediated iron removal from the protein.

Z 010 MOLECULAR RECOGNITION: INTERACTION OF TRANSFERRIN WITH ITS SPECIFIC RECEPTOR,

Anne B. Mason, Department of Biochemistry, University of Vermont College of Medicine, Burlington VT 05405.

Although much information is available on the structure and properties of transferrin and its specific receptor found on the surface of most actively proliferating cells, little is known about the interaction of these two proteins. Our laboratory has sought to investigate which regions of transferrin and its receptor are involved in recognition and binding. Three general approaches have been used. First, holo-transferrin (± iron), individual and combined proteolytically derived half-molecule domains of transferrin (± iron), and holo-receptor have been examined by a number of techniques including gel filtration, sedimentation equilibrium, sedimentation velocity and circular dichroism to estimate molecular size, to measure association and to assess the effects of iron and of pH on secondary structure. Second, binding of iron-59 and/or iodine-125 labeled isologous and heterologous transferrins and individual and combined half-molecule domains to cellular receptors has been measured by kinetic uptake, equilibrium binding and competition experiments. Third, domain specific monoclonal antibodies have been used to probe the interaction of transferrin with its receptor and also to determine the effect of iron on immunological reactivity.

Our work to date with chicken ovotransferrin and chick embryo red blood cells indicates that both domains of ovotransferrin contain receptor recognition sites. In addition, both domains of ovotransferrin must be present, associated and iron loaded to obtain physiological binding to the transferrin receptors on chick embryo red blood cells. Both these studies and work involving human transferrin will be discussed. In the absence of sequencing data for two transferrins of two different species which bind to a common receptor or of an x-ray structure of the transferrin-transferrin receptor complex it is impossible to identify the precise regions of transferrin and the receptor involved in recognition and binding. The three general approaches which we have taken provide a starting point for understanding and defining transferrin-transferrin receptor interaction.

This investigation was supported by USPHS Grant DK/ML-31729

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 011 THE PROTEIN COATS OF FERRITIN AND IRON CORE FORMATION OR DISSOLUTION. J. S. Rohrer, S. P. Sreedharan, M.-S. Joo,* A. Fontaine,† E. Dartyge,† G. Tourillon,† D. E. Sayers,* and E. C. Theil. Departments of Biochemistry and *Physics, North Carolina State University, Raleigh, NC 27695, †Laboratoire pour l'Utilisation du Rayonnement Electromagnetique, Université Paris-Sud, F-91405 Orsay Cedex, France.

Ferritin, the iron storage protein, is a complex of a multisubunit protein coat and an inorganic core of $\leq$4500 Fe atoms as hydrous ferric oxide with various amounts of phosphorus (1). The protein subunits, designated H, M, and L, have highly conserved structures, at least among vertebrates, and may assemble into homopolymeric or heteropolymeric coats; the subunits are the products of several genes which are expressed cell-specifically under the control of environmental (Fe) and developmental signals. Ferritins are distributed widely among plants, animals, and microorganisms, which attests to the importance of segregating and controlling the chemistry of bulk Fe(II) or Fe(III). Experimentally, the protein coats can form iron cores, apparently via nucleation on protein carboxylates (2), from Fe(II) with dioxygen as an electron acceptor; the iron can be released from the cores by reduction, e.g. thioglycolic acid or reduced flavins. Two types of reactions are involved in each case: electron transfer and hydration/dehydration which may or may not be temporally coupled. Analyses of changes in oxidation state deduced from x-ray absorption (XAS) spectra indicate a complex reaction path with heterogeneous rates and the coexistence of Fe(II) and Fe(III) inside the protein coats for periods as long as several hours (3). The significance of variations in subunit structure on core formation and dissolution will be assessed by comparing iron uptake and release in protein coats of two different L subunits and of L subunits genetically engineered to replace carboxylate ligands. [Supported in part by NIH grants DK20251 and GM34675 (E.C.T.) and DOE grant DE-AS05-80-ER10742 (D.E.S.); XAS measurements were made at NSLS, LURE, and SSRL.]

1. Theil, E. C. (1987) Ann. Rev. Biochem. 56:289-315.
2. Yang, C.-Y., Meagher, A., Huynh, B. H., Sayers, D. E., and Theil, E. C. (1987) Biochemistry 26:497-503.
3. Rohrer, J. S., Joo, M.-S., Dartyge, E., Sayers, D. E., Fontaine, A., and Theil, E. C. (1987) J. Biol. Chem. 262:13385-13387.

Role of Iron in the Regulation of Iron Transport and Storage Proteins - II

Z 012 YEAST ACTIVATION HAP1: REGULATION OF DNA BINDING BY HEME, Karl Pfeifer, Kwang Soo Kim, and Leonard Guarente, Massachusetts Institute of Technology, Cambridge, MA 02139.

The yeast *CYC1* gene (iso-1-cytochrome C) is activated by two UASs, UAS1 and UAS2. UAS1 is turned on by HAP1 and UAS2 by the combined action of HAP2 and HAP3. HAP1 is a site specific DNA binding protein which binds to UAS1 and the UAS of the *CYC7* gene (iso-2-cytochrome C), which also requires HAP1 for expression *in vivo*. The activity of *HAP1 in vivo* is induced by heme, as is DNA binding *in vitro*. Oddly, the two sequences bound by HAP1 at UAS1 and the *CYC7* UAS are different. Further, the activity of HAP1 at the two sites differs. UAS1 gives rise to high levels of activity which respond to carbon catabolite repression while the CYC7 UAS activates to a low level of activity which does not respond to carbon source. Recent results to be reported are summarized below.

1) HAP1 encodes a 1482 amino acid protein with a DNA binding region for both UAS1 and the *CYC7* UAS at the extreme amino terminus.

2) The DNA binding region of HAP1 is homologous to GAL4 with a zinc finger structure. Mutating the systeines in the finger abolishes DNA binding.

3) Mutations in the DNA binding domain of HAP1 can selectively knowck out binding to one site.

4) Heme regulation is meditated by a region just carboxyl to the DNA binding domain which inhibits binding in the absence of heme. This region contains a repeated hexapeptide which may bind heme and alleviate the inhibition.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 013 MOLECULAR MECHANISM OF REGULATION OF SIDEROPHORE MEDIATED IRON ABSORPTION, J. B. Neilands, Biochemistry Department, University of California, Berkeley CA 94720.
The availability of a well characterized iron sensing promoter for the biosynthesis and transport operon of the virulence-related siderophore of clinical isolates of Escherichia coli, aerobactin, suggested an examination of the nature of the defect in constitutive mutants. The only such mutant obtained to date is the well known fur (ferric uptake regulation) phenotype, which results in constitutive expression of all iron uptake systems in the bacterium. Expression and isolation of the fur gene product, a 17 kDa ferrous and other divalent metal ion binding protein, has enabled characterization of the operator(s) of the aerobactin operon. The pure Fur protein has been produced in quantity from an inducible expression vector and isolated in a single step by affinity column chromatography over an iminodiacetate agarose support containing coordinated zinc ion. When complexed with a suitable activating divalent metal ion, the Fur protein occupies sequentially primary, secondary and upstream binding sites. The use of DNAasel and hydroxyl radical footprinting techniques has enabled identification of probable contacts between the repressor-metal complex and the operator sequences. Features of the Fur•M(II) binding to the regulatory DNA are apparently novel and appear to explain why operator mutants have, as yet, not been forthcoming. Significantly, only the monoxygenase of the several biosynthetic proteins for aerobactin is localized in the cytoplasmic membrane. It is concluded that the cytoplasm of E. coli constitutes a highly anaerobic milieu favoring the presence of a substantial, but flucuating, pool of hexaaquo or loosely coordinated Fe(II). Thus, siderophore mediated absorption of Fe(III) from the exterior environment is regulated directly at the transcriptional level by an intracellular reservoir of Fe(II) which activates the Fur protein to recognize specific operator sequences. Thus far it has not been possible to hydridize the cloned fur gene to any species other than the wild type of the source organism, E. coli K-12. Further work is required in order to determine if the E. coli model is retained, modified or replaced in other species.

Chemistry and Biology of Metalloregulatory Switches

Z 014 GENETIC AND MOLECULAR BASIS OF COPPER RESISTANCE IN Escherichia coli, J. Camakaris, D. Rouch and B.T.O. Lee, Department of Genetics, University of Melbourne, Parkville Victoria 3052, Australia.
Copper is an essential trace element which is toxic in its "free" ionic form. Mechanisms should exist to ensure that sufficient Cu is available for Cu metalloenzymes whilst avoiding toxicity. Thus Cu, because it is an essential trace element, poses quite different problems to the purely toxic heavy metals such as Hg and Cd.

The study of copper resistance mechanisms has been facilitated by the availability of the plasmid, pRJ1004, which confers an inducible copper resistance in E. coli. Two determinants contribute to the resistance (1) cdr, which encodes DNA repair functions and (2) pCo, which codes for an inducible mechanism which results in decreased intracellular Cu accumulation. The pCo determinant has been cloned and contains at least four genes (pCoA, B, C and R). The pCoC gene product is an inducible 25 Kd Cu-binding protein. The pCoR gene has a trans-acting regulatory role. The roles of pCoA and pCoB are not yet known. Expression of pCo genes is controlled largely at the transcription level. At least two chromosomal gene products are essential for the functions of pCo encoded Cu resistance. Kinetic analysis has shown that the reduced Cu accumulation is due to an energy dependent enhanced efflux of Cu.

We propose that the mechanism of Cu resistance conferred by pCo involves intracellular Cu binding, exclusion by enhanced efflux and chemical modification such that the excluded Cu is not recognised by chromosome encoded Cu uptake systems.

Z 015 METALLOREGULATION IN THE SEQUENCE SPECIFIC BINDING OF SYNTHETIC MOLECULES TO DNA, John H. Griffin, and Peter B. Dervan, Arnold and Mabel Beckman Laboratories of Chemical Synthesis, 164-30, California Institute of Technology, Pasadena, CA 91125.

Interest in the metalloregulation of gene transcription has prompted us to undertake the design, synthesis, and *in vitro* DNA binding studies of a series of hybrid molecules which combine DNA binding subunits with metal binding subunits. Specifically, synthetic analogs of the minor groove binding antibiotic netropsin were linked via oligooxa or oligoaza ionophoric tethers. In addition, the iron chelating moiety EDTA was appended to one end of each molecule in order to study its binding properties by the method of DNA affinity cleaving. Preliminary work showed that one molecule, bis(netropsin)-3,5,7,9,11-pentaoxaheptadecanediamide-EDTA, exhibited strong and specific binding to a DNA restriction fragment only in the presence of Sr^{2+} or Ba^{2+} cations.[1] Recent binding studies on large (plasmid) DNA and on additional restriction fragments have 1) confirmed the metallospecificity for the heavier alkaline earth cations, 2) indicated that the metal-bound ionophoric portion of this molecule binds to GC DNA base pairs in preference to AT base pairs, 3) shown that sites bound strongly in the presence of Ba^{2+} or Sr^{2+} are distinct from the (weak) binding sites observed in the absence of added metal, and 4) shown that the effect is strongly dependent on the structure of the ionophore, in that lower and higher homologs show little, if any, metalloregulation. The results are consistent with a model in which specific DNA phosphate:dication:ionophore complexes are formed adjacent to AT rich netropsin binding sites in the minor groove of B DNA, increasing the binding energy. In an effort to alter the metallospecificity, the synthesis of nitrogen-containing analogs has recently been completed. DNA binding properties of these molecules, which are expected to bind "softer" transition and post-transition metals, will be reported.

[1]Griffin, J.H.; Dervan, P.B. *J. Am.Chem. Soc.*, **1987**, *109*, 6840.

Z 016 THE MerR METALLOREGULATORY PROTEIN: PROMOTER AND RNA POLYMERASE INTERACTIONS WITH A METAL-RESPONSIVE POSITIVE ACTIVATOR OF GENE EXPRESSION, T. O'Halloran, M.Chael, B.Frantz, D.Ralston, M.Shin, J.Wright, L. Xue. Dept. of Chemistry, Northwestern University, Evanston, IL 60208.

Metalloregulatory proteins, factors which mediate metal responsive gene expression, play key roles in translating inorganic signals into changes in cellular activity. We now show that the repression and metal responsive positive activation of bacterial mercury resistance genes (TN501) are mediated by a single such protein, namely MerR. In vitro transcription run-off experiments demonstrate that the purified mercuric ion/MerR protein complex is a positive activator of mercury resistance genes (merTPAD), whereas the mercury free protein is a repressor. Mercuric ion concentrations in the range observed to induce mercury resistance in vivo (10^{-6}-10^{-8}M) increase in vitro transcription from the metal-responsive promoter over a hundred fold. MerR protein interactions at the overlapping and transcriptionally divergent merR and merTPAD promoters, the latter being the only metal responsive one, have been probed by DNase, DMS and hydroxyl radical protection and ethylatio interference. Models suggest the protein primarily contacts one face of a B-form DNA helix, and makes a few weaker contacts on the opposite face at th center of the palindrome. While the overall affinity of the protein for DNA decreases 2-10 fold in the presence of micromolar mercury, protection is unchanged in the presence and absence of mercury. Gel mobility and DNA footprinting analysis show the MerR protein is capable of weakly inducing intermediate complexes between the metal-responsive promoter and the enzyme RNA Polymerase (RNAP) in the absence of mercury. Stable, heparin resistant and transcriptionally competent MerR/RNAP complexes of the metal responsive promoter can only be formed in the presence of mercuric ion. Protection patterns indicate this series of induced complexes are analogous to 'open' and 'initiated' promoter complexes and contain unique MerR footprint features. A model proposed for this activation event includes specific contacts within a ternary complex consisting of metalloregulatory protein, DNA and RNAP which cannot proceed to an transcriptionally initated complex until some of contacts are altered by mercuric ion coordination to MerR.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 017 PLASMID-DETERMINED HEAVY METAL RESISTANCES: Arsenic, cadmium and mercury, Tapan K. Misra and Simon D. Silver, Department of Microbiology and Immunology, University of Illinois, Chicago, IL 60680.

Bacterial resistances to heavy metals are often governed by genes on plasmids and transposons. The arsenic and mercury resistance operons are inducible; the cadA system for Cd^{2+} resistance is constitutive. Bacteria accumulate arsenate via the chromosomal-gene governed phosphate transport systems and resistant cells then efflux arsenate and arsenite by an ATPase arsenic pump. The structural genes, arsA, arsB, arsC, for the ars operon of Escherichia coli plasmid R773 have been identified from DNA sequencing analysis. The ArsA protein has two putative adenylate binding sites homologous to those in nitrogenase and the β subunit of the oxidative phosphorylation ATPase. The ArsB protein is an integral membrane protein. ArsA and ArsB proteins form a complex with arsenite pumping activity. ArsC protein confers additional arsenate resistance and efflux (1). Adenylate-binding regions homologous to those described above have also been identified in Mdr multidrug resistance glycoproteins associated with tumor cells that have become refractory to chemotherapy presumably by pumping out a variety of structurally unrelated compounds from the cells. The DNA sequence of the cadA cadmium-resistance determinant from Staphylococcus aureus plasmid pI258 has been determined. One open reading frame would encode for a polypeptide of 727 amino acid with significant amino acid identities when compared with the sequences of K^+ ATPases of bacteria and other E1E2 cation translocating ATPases of microbial and animal cells such as the Na^+/K^+ ATPase and the muscle Ca^{2+} ATPase. The catalytic sites where adenylate binds, and the aspartyl residue that is phosphorylated during catalytic cycle are highly conserved in these ATPases. The mer operon for the mercurial resistances contains 5 to 7 genes. The first mer genes encode proteins with Hg^{2+} transport function. In absence of this function, the cells are Hg^{2+} sensitive. The enzyme mercuric reductase (governed by the merA gene) is a redox-active flavo-protein that reduces Hg^{2+} to Hg^0. Hg^0 is less toxic and volatile. The mercuric reductase has significant sequence homology with a variety oxido-reductases of bacterial and mammalian sources, including human glutathione reductase (2). Besides mercuric reductase, the enzyme organomercurial lyase (governed by the merB gene) breaks the C-Hg bond in organomercurials such as phenylmercury. The regulation of mer structural genes is significantly different among various gram positive and gram negative bacteria.

1. C.-M. Chen, T.K. Misra, S. Silver and B.P. Rosen (1986) J. Biol. Chem. 261, 15030.
2. T. Misra, N. Brown, L. Haberstroh, A. Schmidt, D. Goddette and S. Silver (1985) Gene 34, 253.

Metallothioneins

Z 018 METALLOTHIONEINS: STRUCTURE AND METAL BINDING PROPERTIES, Ian M. Armitage, Bruce A. Johnson, and J.A. Malikayil, Yale University, Department of Molecular Biophysics and Biochemistry, 333 Cedar Street, New Haven, CT 06510.

Research on metallothioneins is entering its fourth decade [J. Amer. Chem. Soc. 79:4813(1957)]. However, a great deal remains to be known about this proteins's biological function in the metabolism of essential metals and the sequestration of toxic metals. Detailed studies using both NMR and X-ray crystallography have established the metal-ligand coordination geometry and peptide fold for Cd(II) and Zn(II) in two mammalian MTs. We will report progress in establishing similar structural information on MTs from two other species and the dynamics and coordination properties of mammalian MTs with different metals. In particular, information on Hg(II) binding to rabbit liver MT, and progress in the structure determination of yeast (Saccharomyces cerevisiae) and Neurospora crassa will be described.

UV spectroscopic studies of Hg(II) binding have demonstrated that Hg(II) binds initially by replacing Cd(II) with retention of tetrahedral geometry, but subsequently adopts a linear coordination geometry. Evidence will be presented that is consistent with metal exchange between Hg(II) and Cd(II) MTs resulting from bimolecular collisions between the two protein species.

Yeast and Neurospora MTs provide two unique examples of Cu coordination stoichiometries, with yeast MT binding 8 Cu(I) ions through 12 cysteine thiolate ligands, and Neurospora binding 6 Cu(I) ions through 7 cysteine thiolate ligands. The complete sequential assignments of the ^1H NMR spectra in both these MTs are underway. The size of the Neurospora MT and the copper coordination of both these MTs provided interesting complications in the determination of the 3D structure using NMR methods. We will report our progress towards the 3D structures of these two proteins and their relationship to the presumed functional properties and evolution of this protein. (Supported by NIH Grant DK 18778).

Z 019 SOLUTION STRUCTURE DETERMINATION AND PATHWAY OF CLUSTER FORMATION IN METALLOTHIONEIN, Milan Vašák, Biochemisches Institut der Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland
The solution structure of ^{113}Cd enriched Cd_7-metallothionein-2a (MT) from rabbit liver has been calculated from a combination of two-dimensional (2D) homonuclear (^{1}H) and heteronuclear (^{1}H - ^{113}Cd) nuclear magnetic resonance (NMR) data using a distance geometry algorithm (1-3). The structure revealed two domains encompassing 3 and 4 Cd(II) ions organized in two tetrahedral tetrathiolate clusters. Although the recently published crystal structure of native (Zn_2,Cd_5)-MT from rat liver displays a similar cluster organization (4) only ca. 25 % of the sequence specific metal-cysteine connectivities are identical in the two models. To exclude species differences and preparative artefacts as the possible source of these unequal cluster topologies, we have now carried out 2D heteronuclear NMR studies also on reconstituted $^{113}Cd_7$- and on native (Zn_2, $^{113}Cd_5$)-MT of rat liver. These results showed that the cluster topologies of reconstituted $^{113}Cd_7$-MTs of rat and rabbit are identical. The NMR spectra of the <u>in-vivo</u> labeled (Zn_2, $^{113}Cd_5$)-MT indicate the existence of several forms differing in distribution of the metal ions. Of these a major form contains $^{113}Cd_4$-cluster topology also found in reconstituted $^{113}Cd_7$-MTs (5). In an independent study the pathway of cluster formation in rabbit MT has been investigated by ^{113}Cd NMR spectroscopy at pH 7.2 and 8.6. Stepwise incorporation of ^{113}Cd at pH 7.2 results in NMR features consistent with the cooperative cluster formation the C-terminal Cd_4-cluster being formed first. In contrast, at pH 8.6 the first 4 ^{113}Cd appear to be bound to separate CdS_4-sites which undergo chemical exchange with partially formed cluster(s). Further ^{113}Cd addition generates sequentially the Cd_4- and Cd_3-clusters in a cooperative manner. The unequal cluster stability allows for selective cluster filling. Indeed, in the mixed [$Co(II)_3$, $^{113}Cd_4$]-MT form, as judged by ^{113}Cd NMR and electronic absorption spectra, Cd(II) ions occupy the four-metal cluster and Co(II) ions three-metal cluster.

<u>References:</u> (1) M.H. Frey et al., J.Amer.Chem.Soc.<u>107</u>, 6847 (1985) (2) G. Wagner et al., Eur.J.Biochem.<u>157</u>, 275 (1986) (3) A. Arseniev et al., J.Mol.Biol. (1988), in press (4) W.F. Furey et al., Science <u>231</u>, 704 (1986) (5) M. Vašák et al., J.Mol.Biol. <u>196</u>, 711 (1987)

Banquet Address

Z 020 MOLECULAR BASIS OF BACTERIAL RESISTANCE TO MERCURIALS, Christopher T. Walsh, Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.
Regulatory and structural genes of the Mer operon confer resistance to organomercurial and inorganic mercuric salts to bacteria that harbor these genes. Studies in progress on the mer R gene product (metalloregulatory DNA binding protein), mer b gene product (organomercury lysase), and mer A gene product will be discussed.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Metallothionein and Metallothionein-like Peptides

Z 021 PHYTOCHELATINS IN PLANTS, Erwin Grill
Lehrstuhl für Pharmazeutische Biologie, Universität München, FRG
Phytochelatins are a class of heavy metal-binding peptides isolated from several dicotyledonous and monocotyledonous plants. These peptides consist of repetitive γ-glutamylcysteine units with a carboxyl-terminal glycine and range from 5 to 23 amino acids in length. Phytochelatins are induced by cations of e.g. cadmium, zinc, copper, lead, mercury and silver, but also by anions like arsenate and selenite. The intracellular distribution of some of these heavy metals in plant cell suspension cultures revealed an almost exclusive chelation of the toxic ions by phytochelatins. This finding and the fact that the peptides are synthesized in plants ranging from the phylogenetically primitive algae to the highly advanced orchids, point to the principal function of phytochelatin peptides in the heavy metal sequestration of plants.
Their cellular function is assigned to detoxification and homeostasis of heavy metal ions. A detoxifying role could be concluded from experiments (1) inhibiting phytochelatin biosynthesis, (2) monitoring the interaction of toxic effect and peptide formation and (3) by overproduction of phytochelatins. A homeostatic function could be perceived by the ability of physiological concentrations of zinc and copper employed for cultivating plants and their cell cultures to induce phytochelatins. The peptidic zinc- and copper-complexes may serve as a degradable storage form in the regulation of the plants cellular zinc and copper supply.
On account of the repetitive γ-glutamyl linkages in the primary structures phytochelatins are no primary gene products. An involvement of glutathione biosynthesis in phytochelatin formation was demonstrated by specific blocking of glutathione's biosynthetic pathway, by analysing the incorporation of γ-glutamylcysteine units into the peptides, and by isolation of homo-phytochelatins. Homo-glutathione (γ-Glu-Cys-ß-Ala) containing plants like soybean synthesize homo-phytochelatins, phytochelatins in which the carboxyl-terminal glycine is replaced by ß-alanine.
Phytochelatins and metallothioneins both complex heavy-metal ions by thiolate coordination, but differ completely in their biosynthesis. Thus, an evolutionary divergence in heavy metal sequestration has occured betweeen animals and plants.

Z 022 NEUROSPORA COPPER METALLOTHIONEIN: MOLECULAR STRUCTURE AND REGULATION OF GENE EXPRESSION, K. Münger, U.A. Germann, M. Beltramini and K. Lerch, Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.
In the presence of nontoxic doses of $CuSO_4$ Neurospora crassa accumulates the metal with the concomitant synthesis of a low molecular weight copper-binding protein. The molecule which consisits of 25 amino acids, binds 6 Cu ions, and shows extensive sequence similarity to the amino-terminal part of vertebrate metallothioneins. Absorption, circular dichroism, and EPR spectroscopy of N. crassa Cu metallothionein indicate the copper to be bound to cysteinyl residues as a Cu(I)-thiolate cluster $(Cu(I)_6RS_7)^-$ (1). X-ray absorption edge and EXAFS spectroscopy data are most consistent with a three- or four-coordinate geometry around the Cu indicating a compact and rigid cluster structure for N. crassa Cu metallothionein (2). Upon excitation in the UV the protein emits an unusual luminescence characterized by a large Stokes shift with a maximum at 565 nm (2). The luminescence emission - attributed to a triplet excited state of the Cu(I)-to-sulfur charge transfer complex - has a lifetime of 10.3 µs. In agreement with the known amino acid sequence of N. crassa metallothionein, the recently cloned gene codes for a polypeptide of 25 amino acids (3). The promoter region of the gene is devoid of sequences related to the highly conserved metal regulatory elements of mammalian metallothionein genes or the copper responsive elements of the yeast metallothionein gene. Unlike the mammalian metallothionein genes which are induced by different metal ions, (Cu^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}), the transcription of the N. crassa metallothionein gene is strictly copper dependent (4). In cultures of N. crassa inoculated and grown in copper-supplemented media, metallothionein m-RNA appears during the late logarithmic growth period and is detectable for more than 30 h. In response to copper shock, however, rapidly increasing amounts of metallothionein m-RNA are detected within minutes after copper administration at any time in vegetatively growing mycelia of N. crassa. Maximum levels are detected about 1h after addition of copper to the medium. The half-life time of the m-RNA is approximately 2.5 h. The amounts of copper metallothionein reach a maximum level at 3h after induction and thereafter remain constant. The rapid induction by copper ions of metallothionein m-RNA and metallothionein together with the remarkable stability of the native protein intracellularly suggests that this protein serves an important homeostatic role in the copper metabolism of this fungus.

1) M. Beltramini and K. Lerch (1986), Environ. Health Perspect. 65, 21-27.
2) T. A. Smith, K. Lerch, and K. O. Hodgson (1986), Inorg. Chem. 25, 4677-4680.
3) K. Münger, U. A. Germann, and K. Lerch (1985), EMBO J. 4, 2665-2668.
4) K. Münger, U. A. Germann, and K. Lerch (1987), J. Biol. Chem. 262, 7363-7367.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 023 STRUCTURAL ASPECTS OF METAL-γ-GLUTAMYL PEPTIDES, Dennis R. Winge, R. Neil Reese, Rajesh K. Mehra, E. Bart Tarbet and A. Hughes, University of Utah Medical Center, Salt Lake City, Utah 84132.

γ-Glutamyl peptides of the general structure $(\gamma\text{-Glu-Cys})_n$-Gly were isolated from Schizosaccharomyces pombe grown in the presence of Cu or Cd salts. The peptides within the metal-thiolate clusters ranged in length from 5 to 13 residues differing in the number of γ-Glu-Cys dipeptide repeats. In several preparations of γ-Glu peptides the predominant peptides were of n_3 and n_4. Some peptides were present in the desGly form. Each metal-γ-Glu peptide complex exists as a oligomer, but the peptide stoichiometry within a complex is unclear. The complex formed with Cu salts is clearly a Cu(I)-thiolate cluster with the metal center shielded from solvent. Cu complexes can form with peptides of unique lengths, but complexes of highest metal binding stoichiometry and greatest stability are formed with mixtures of peptide lengths. The Cd-γ-Glu peptide complexes contain labile sulfur unlike the Cu complexes. Heterogeneity exists in the labile sulfur content of the Cd clusters. Complexes with the highest sulfide content exhibit a characteristic 300-310nm absorption band. Cd-γ-Glu peptide complexes of low sulfide content or devoid of sulfide can be reconstituted with sulfide to generate the 300nm absorption band. It appears that each Cd-thiolate cluster contains a single sulfide and that the basic oligomer can further associate to yield higher molecular weight species. Sulfide addition results in the association of oligomers, the sulfides may bridge oligomers together. High sulfide forms of the Cd-thiolate cluster imparts greater stability to the complex. In addition to the accumulation of sulfide in Cd-γ-Glu peptide complexes, S. pombe secretes sulfide into Cd-containing media resulting in insoluble Cd-S formation. The γ-Glu peptide system is the major mechanism of metal tolerance in S. pombe. Saccharomyces cerevisiae exhibits tolerance to only Cu salts by induction of metallothionein-like polypeptide. In a separate yeast the γ-Glu peptide system is induced by Cd salts, but Cu salts elicit formation of two metallothionein-like polypeptides.

Physiology and Diseases of Unbalanced Metal Homeostasis

Z 024 A POSSIBLE ROLE FOR INTRACELLULAR CU-BINDING PROTEINS IN CU-UPTAKE, F. Palida, G. Waldrop, and M. Ettinger, Department of Biochemistry, SUNY at Buffalo, Buffalo, NY 14214.

The kinetics of Cu(II)-transport by hepatocytes (Km 11 ± 0.6 μM, Vmax 2.7 ± 0.6 nmol/min/mg protein) and fibroblasts (Km 6.9 ± 0.2 μM, Vmax 2.6 ± 0.1 nmol/min/mg protein) are similar. Transport is passive in both directions. Albumin markedly decreases initial rates of Cu-uptake with both cell types by decreasing the effective, extracellular Cu-concentration. As equilibrium is approached (3 h), hepatocytes compete with albumin much more effectively than fibroblasts do. Thus, at 10 μM ^{64}Cu(II) plus 10 μM albumin, fibroblasts accumulated only 10% as much ^{64}Cu as at 10 μM ^{64}Cu(II) alone, but hepatocytes accumulated approximately the same amount of ^{64}Cu with or without albumin. Neither cell type takes up ^{125}I-albumin. Sephadex G150 (1.5 x 100 cm) profiles show that hepatocytes contain a 38 kd cytosolic, Cu-binding-protein fraction which is not detected in fibroblasts. This may account, in part, for the observed differences in net Cu-uptake by hepatocytes and fibroblasts at equilibrium. Menkes disease is a fatal, X-linked disease of Cu-metabolism. Fibroblasts from the brindled mouse mutant model of Menkes disease accumulate excess Cu from albumin-containing media. These fibroblasts contain elevated levels of metallothionein (MT) to which the excess Cu binds. Comparable levels of MT can be attained in normal fibroblasts by Cu,Zn or dexamethasone induction. Cu-uptake by normal fibroblasts with elevated MT-levels is normal, i.e., the brindled/Menkes phenotype of excess Cu-accumulation is not exhibited by these cells. This implies a role for the protein associated with the primary defect in the mutant in determining net Cu-uptake by fibroblasts. A cytosolic protein has been identified by HPLC and SDS-PAGE which appears to be abnormally low in the hepatic and renal cytosols from the brindled mice. These results taken together, suggest a role for non-MT cytosolic Cu-binding proteins in determining net Cu-uptake.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 025 Cellular Accumulation of Iron and Other Transition Metals by a Transferrin Independent Uptake System

Jerry Kaplan, Anne Sturrock, James Alexander, (Department of Pathology and Department of Medicine, University of Utah Medical Center, Salt Lake City, Utah 84132

A number of studies demonstrate an uptake system for iron independent of the iron transport protein transferrin. Among the most persuasive evidence is the existence of a line of mice exhibiting a mutation in the transferrin gene resulting in severe hypo-transferrinemia. Homozygotes for this disorder are severely anemic yet at the same time exhibit iron overload disease of the liver, pancreas, and heart. A phenocopy of this disorder can be established in normal animals by overloading the ability of transferrin to bind iron; at which point iron molecules introduced into plasma will be cleared by the transferrin-independent uptake system. Studies employing cultured cells also demonstrate the presence of the transferrin-independent iron uptake system. A number of different experiments indicate that uptake does not involve endocytosis and most probably results from a carrier mediated transport system. Cells unable to bind transferrin because of proteolysis of transferrin receptors are still capable of accumulating iron salts, "ionic iron". Treatment of cells with sulfhydryl reagents prevents endocytosis and accumulation of transferrin bound iron but has no inhibitory effect on accumulation of ionic iron. This transport system is capable of accumulating other metals, and may be an uptake system for transition metals. Accumulation of ionic iron is blocked by a variety of other metals including Cu, Zn, Mn and Cd. Simultaneous incubation of HeLa cells with ionic iron and Cd can alleviate the toxic effect of Cd. Whereas, prior incubation of cells with ionic iron, or simultaneous incubation with diferric transferrin and Cd was without effect. Ionic iron could competitively inhibit the uptake of Cd^{109}. Conditions which result in an increase in the uptake of ionic iron show a similar increase in Cd^{109}. These results suggest the existence of a transport system capable of mediating the accumulation of a number of different transition metals. (Supported by funds from the National Institute of Health.)

Phytochelatins in Plants

Z 026 Biochemical Basis of Cadmium Resistance and the Role of Metal-Binding Peptides $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ in Plant Cells.

John C. Steffens, Department of Plant Breeding, Cornell University, Ithaca, NY 14853

Tomato cell cultures selected for growth on normally lethal concentrations of $CdCl_2$ synthesize the metal binding polypeptides $(\gamma\text{-Glu-Cys})_3\text{-Gly}$ and $(\gamma\text{-Glu-Cys})_4\text{-Gly}$. Cd^R cells accumulate these peptides in higher amounts than Cd^S cells when challenged by Cd. The pool of precursor γ-Glu-Cys increases five-fold over the same pool in Cd^S cells. Specific inhibition of γ-glutamylcysteine synthetase by buthionine sulfoximine prevents accumulation of these peptides and abolishes resistance to Cd. Similarly, BSO sensitizes Cd^S cells to previously nontoxic levels of Cd. Growth of Cd^R cells is unaffected under these conditions. In the absence of Cd, Cd^R cells are resistant to growth inhibition by concentrations of BSO which inhibit growth of CD^S cells, indicating that the activity of the target enzyme may be altered in Cd^R cells. Assay of γ-glutamylcysteine synthetase from both cell lines shows that activity of this enzyme in Cd^R cells is about four-fold higher than in Cd^S cells.

Cd^R cells are not cross-resistant to other heavy metals, and recent work suggests that metal detoxification by these peptides may be Cd-specific. Low levels of these peptides are present in cells in the absence of heavy metals, and the occurrence of acid-labile sulfur as a component of the metal complex raises questions concerning the possible role of these peptides besides that of Cd detoxification. The most likely role appears to be as a substrate for APS sulfotransferase, to serve as a carrier for sulfur during its reduction from sulfate to the level of cysteine.

Z 027 CHARACTERIZATION OF ZN-BINDING PEPTIDES ASSOCIATED WITH A DECLINE DISORDER OF CITRUS.

Kathryn C. Taylor, L.Gene Albrigo and Christine D. Chase, University of Florida, Gainesville FL 32607.

Citrus blight is a decline disorder of unknown etiology. The earliest symptom of the disorder is a redistribution of Zn within the tree. The canopy exhibits symptoms of Zn deficiency while Zn accumulates in the phloem. The phloem Zn is associated with a complexing agent.

A 4 kilodalton, highly anionic, zinc binding fraction was isolated from phloem tissue of blight-affected 'Valencia' sweet orange (Citrus sinensis (L.) Osbeck) and 'Marsh' grapefruit (C. paradisi Macf.) trees. QAE-Zeta chromatography revealed the Zn binding fraction contained at least four different anionic species. Two of the four species were present in tissue from healthy as well as blight-affected trees. The other two species were found in only the blight-affected trees. Amino acid analysis was performed on each of the partially purified Zn-binding factors. The four factors had very similar amino acid compositions and contained a high percentage of asp/asn residues, indicating they are not poly(gamma-glutamyl-cysteinyl)-glycine peptides. Further characterization of these peptides and their metal binding properties is underway.

Z 028 RESPONSES OF PLANT CELLS TO CD EXPOSURE

G. J. Wagner, Plant Physiology/Biochemistry/Molecular Biology Program, Agronomy Department, University of Kentucy, Lexington, KY 40546-0091

Higher plants and plant cultured cells respond to challenge with high concentrations of Cd by forming non-metallothionein-like, Cd-binding peptides, also called phytochelatins. In cultured cells rendered unable to form Cd-peptide (BSO inhibited), but not in uninhibited cells, challenge with high Cd concentration results in decreased growth which is correlated with exposure concentration. These and other results suggest a Cd-detoxification role for Cd-peptide in plants, at least those exposed to high levels of Cd. For Cd-peptide versus metallothionein, modes of ''induction'', synthesis, and specificity of metal binding and ''induction'' differ significantly. Zinc does not appear to bind to or induce significant Cd-peptide ligand. Comparative properties of Cd peptide and metallothionein will be discussed.

Even after very high Cd challenge ($\leq$400µM), tobacco cultured cells require 1 to 2 hours to form significant levels of Cd peptide, yet cells are shown to equilibrate with metal (also Zn) immediately. Thus, the question arises as to how cells accommodate the presence of intracellular Cd before significant Cd peptide is formed. Studies of organic acid-to-Cd and Zn stoicheometry, vacuolar compartmentation of Cd and Zn, and of organic acid biosynthesis support the hypothesis that Cd and Zn may be rapidly sequestered in plant vacuoles as organic acid metal complexes, conferring initial tolerance. Results of studies comparing the occurrence of Cd peptide versus Cd-metallothionein in transgenic tobacco plants expressing mouse metallothionein will also be described.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Gene Regulation

Z 100 BACTERIAL AND FUNGAL TRANSPORT SYSTEMS SPECIFIC FOR MANGANESE George Auling, Institute of Microbiology, University of Hannover, D-3000 Hannover 1, Schneiderberg 50, FRG
The existence of a high-affinity, energy-dependent manganese influx in Brevibacterium ammoniagenes ATCC 6872 and in Aspergillus niger ATCC 11414 was demonstrated. In Brevibacterium ammoniagenes ribonucleotide reduction, the central step in DNA precursor biosynthesis is known to be manganese dependent. Comparison of purified membranes prepared from manganese sufficient and deficient cells revealed that 14 out of 66 polypeptide bands detectable by SDS-PAGE changed in intensity upon manganese depletion. In the presence of manganese similar quantitative alterations of the protein composition of the membrane were induced when ribonucleotide reduction was inhibited by hydroxyurea or when DNA replication itself was inhibited by mitomycin C. The possibility is discussed that one or more of the polypeptides enhanced in the membrane of manganese deficient cells of B. ammoniagenes represent the derepressed manganese carrier of the high-affinity manganese transport system.

Z 101 TRANSCRIPTION FACTOR(S) WHICH BINDS TO THE UPSTREAM ACTIVATOR SEQUENCES OF THE YEAST COPPER METALLOTHIONEIN GENE, Lawrence W. Bergman and Cynthia R. Timblin, Ohio University, Athens, Ohio 45701.
The yeast copper metallothionein gene (CUP1) is transcriptionally regulated in response to the presence of copper in the growth medium. Although the cis-acting elements responsible for the response are known, very little is known of the trans-acting factors involved in CUP1 expression. A promoter fusion between the CUP1 upstream activating sequences and the regulatory sequences of the yeast PHO5 gene was used to demonstrate that efficient transcriptional regulation in response to copper is mediated by DNA sequences contained on a 101 bp HinfI-HinfI CUP1 restriction fragment. An in vitro approach was taken to detect specific protein(s) binding to the upstream activating sequences of the CUP1 gene. DNA binding activity(s) specific for these sequences has been purified 11,000-fold from yeast nuclear extracts by Heparin Agarose and CUP1-specific DNA-cellulose chromatography. Nucleoprotein gel electrophoresis reveals two discrete specific protein-DNA complexes. DNAase I footprinting detects two regions of protection, region I and II, which includes the repeated consensus sequence 5'-TCTTTTTGCT-3' which is implicated in the transcriptional response to copper.

Z 102 PURINERGIC AGONIST MEDIATED INDUCTION OF RAT HEPATIC ZINC METALLOTHIONEIN. Frank O. Brady, Scott H. Garrett and Koji Arizono, Department of Biochemistry, University of South Dakota School of Medicine, Vermillion, SD 57069 USA.
Purinergic agonists cause activation of either adenylate cyclase (P_1) or phospholipase C (P_2) by adenosine (AMP) and ATP (ADP), respectively. Rat liver possesses the A_2 subtype of the P_1 receptor and the P_2y subtype of the P_2 receptor. In vivo injections of ATP (10 μmoles/kg) or adenosine (100 μmoles/kg) were administered to male, Sprague-Dawley rats (250-300 g), and the levels of hepatic zinc metallothionein (MT) were assessed. ATP (single or three injections) did not elevate ZnMT levels above controls receiving saline injections, but adenosine (three injections at 0, 4, and 8 hr) did (3.4-fold induction). Various agonist analogues of adenosine are available, and these were studied in vivo. 2-Chloroadenosine (100 μmoles/kg) and 5-N-ethylcarboxamide adenosine (NECA) (10 μmoles/kg) were effective in vivo inducers at 11 hr after single injections with 7.3- and 5.5-fold inductions, respectively. 5'-Chloro 5'-deoxyadenosine (50 μmoles/kg) were modestly effective after a single injection (2.1-fold) and better after three injections (3.9-fold). A time course study with 2-chloroadenosine (100 μmoles/kg) revealed near maximal induction at 11 hr with a continuing rise to 25 hr. A dose response curve (11 hr) revealed an $ED_{0.5}$ for 2-chloroadenosine of 20 μmoles/kg. Theophylline pretreatment can block the induction by 2-chloroadenosine. The in vivo studies have been confirmed in vitro, using isolated, cultured rat hepatocytes. The multihormonal modulation of the induction of rat hepatic ZnMT now includes purinergic agonists which activate adenylate cyclase. (Supported by a research grant from the Parson's Fund)

Z 103 POST-TRANSCRIPTIONAL CONTROL OF GLUTATHIONE PEROXIDASE GENE EXPRESSION BY SELENIUM.

Sunil Chada, Constance Whitney and Peter Newburger, University of Massachusetts Medical Center, Worcester, MA 01605.

Selenium is an essential element in the mammalian diet and has been shown to have a protective effect in animal models of carcinogenesis. The only well characterized selenoprotein is Glutathione Peroxidase (GSH-Px), in which the selenium is incorporated as an unusual selenocysteine residue. This enzyme is important in protecting cellular membranes and DNA from peroxidative damage.

We have isolated cDNA clones encoding the entire human GSH-Px mRNA, and have shown that the selenocysteine residue is encoded by an opal terminator codon **TGA**. This is the first example of a termination codon being suppressed in human cells.

We have developed a model system with which to study selenium regulated gene expression using a human promyelocytic leukemia cell line (HL60), which may be grown in a defined medium with or without selenium. Cellular GSH-Px activity and steady-state protein levels fell sharply when selenium was removed from the media, whereas steady-state mRNA levels and transcription rates were independant of the presence or absence of selenium. This indicates that GSH-Px gene expression is regulated post-transcriptionally by selenium, probably by the availibility of selenocysteine.

Z 104 REGULATORY SEQUENCES IN HUMAN A FERRITIN H GENE,

Panos Papadopoulos, Elisabetta Zappone and Jim Drysdale, Tufts University School of Medicine, Boston MA 02111.

Ferritin H synthesis is induced by several factors including heavy metals, hormones and growth factors, and by differentiation or proliferation. We have obtained additional sequence from the promoter region of a functional human H ferritin gene and find many sequences that affect the transcription of several other genes. In addition to several GC boxes in the 5' upstream region and in the first intron, there are sequences with complete homology to regulatory elements in genes for globin, transferrin receptor, heat shock proteins, interferon, c-fos and interleukin. There is extensive homology with the promoters of the oncogenes c-erbB-1 and c-erbB-2 and several copies of sequences required for basal and cadmium induced synthesis of metallothionein. Since ferritin H mRNA levels are also induced by cadmium (experiments with M. Karin), ferritin H and metalliothionein may use the same metal regulatory element. Although induced by phorbol esters, we do not find any of the known phorbol responsive elements in ferritin H.

Z 105 A NOVEL DNA BINDING METALLOPROTEIN FROM (+Zn) AND (-Zn) *E. GRACILIS*,

Kenneth H. Falchuk, Marta Czupryn and Bert L. Vallee, Harvard Medical School, Boston, MA 02115.

The existence of gene regulatory zinc metalloproteins in *E. gracilis* was postulated to account for the effects of zinc, and its deficiency, on the differential activation or repression of specific sets of genes in this organism (Vallee and Falchuk, 1981, *Phil. Trans. R. Soc. Lond.* B 294:185). We have now purified a chromatin metalloprotein, examined its effects on transcription and studied the consequences of zinc deficiency on its metabolism. In (+Zn) *E. gracilis*, it has a molecular weight of 10,000 and contains 1.1 - 1.4 g-atom Zn/mole, but no Cu, Fe, or Mg. It binds tightly to single stranded and weakly to double stranded DNA. It enhances RNA synthesis by the α-amanitin sensitive RNA polymerase II from both *E. gracilis* and wheat germ. In contrast, it does not affect the activity of the α-amanitin resistant RNA polymerase fraction. In (-Zn) cells, the molecular weight and amino acid composition of the protein are unchanged. Remarkably, instead of Zn, it contains 1.4 - 1.6 g-atom Cu/mole. The Cu protein binds weakly to both single and double stranded DNA. The replacement of Zn by Cu in this protein opens the way to examine the role of the metal in the function of it, and perhaps other DNA binding metalloproteins. This may provide information critical to understanding the control of gene activity by zinc and the biological effects induced by zinc deficiency.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 106 STUDIES ON THE REGULATION OF HEPATIC CERULOPLASMIN GENE EXPRESSION, Jonathan D. Gitlin, Washington University School of Medicine, St. Louis, Mo 63130.
Ceruloplasmin is a serum α -2 glycoprotein which contains greater than 95% of the copper found in the plasma of vertebrate animals. This protein is an acute-phase reactant and has diverse biologic roles which include copper transport and metabolism, anti-oxidant metabolism, and angiogenesis. We have isolated human ceruloplasmin cDNA clones and used these to study the molecular mechanisms regulating hepatic ceruloplasmin gene expression during inflammation. In all species examined, hepatic ceruloplasmin mRNA content increases 6-10 fold within 24 hours following an initial stimulus. Human liver contains two ceruloplasmin specific mRNAs (4.2 & 3.7kB), present in equivalent amounts, and following inflammation there is an equivalent increase in the hepatic content of both transcripts without a change in transcript size. Nuclear run-on assays indicate that this increase in hepatic ceruloplasmin mRNA content is largely the result of an increase in the relative rate of ceruloplasmin gene transcription although changes in ceruloplasmin mRNA turnover are also involved. Studies in human hepatoma-derived cell lines indicate that the increase in ceruloplasmin gene transcription is mediated by several cytokines including interleukin-1 and tumor necrosis factor. Ceruloplasmin gene expression or protein synthesis was not affected in these studies by estrogen or copper ion concentration suggesting that the reported effects of these compounds on ceruloplasmin serum concentration occurs by post-translational mechanisms.

Z 107 IN VIVO FOOTPRINTING OF MerR BINDING TO THE mer REGULATORY REGION, A. Heltzel, P. Totis and A.O. Summers, University of Georgia, Athens, Georgia 30602.
Expression of the structural genes (merTPCAD) of the bacterial mercury resistance locus is regulated by the merR product, a DNA binding protein which acts as a repressor in the absence of and as an activator in the presence of mercury. MerR also negatively regulates its own sythesis. The mer regulatory region includes the in vitro MerR binding site, a symmetrical sequence with 7-bp inverted repeats separated by 4-bp, and two divergently oriented promoters, P_R and P_{TPCAD}. In the presence of MerR, in vivo methylation of uninduced and Hg-induced cells shows that, regardless of mercury being present, four out of nine guanine residues in the symmetrical dyad are protected. However, the reactivity of DNA from Hg-induced cells reveals that at least three additional guanine residues in the symmetrical site are altered consistent with a shift of MerR binding or with RNA polymerase binding upon activation of P_{TPCAD}. Different reactivities of guanine residues in the -10 and -35 regions of P_{TPCAD} in Hg-induced cells suggest RNA polymerase occupation at P_{TPCAD} upon activation. In vivo experiments extending further into the merR reading frame indicated no additional MerR binding sites. Our current model is that MerR binds to the same general region as an activator or as a repressor. Upon induction with mercury, the activator conformation of MerR bound at the dyad structure allows RNA polymerase binding to P_{TPCAD}. We are currently investigating repression- or induction-deficient as well as merR deletion mutants using the above approach.

Z 108 DNA METHYLATION IS CORRELATED TO REGULATION OF HUMAN MT IF, MT IG AND MT IIA GENES. N. Jahroudi, G. Bietel, C. Sadhu and L. Gedamu. Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada.
The expression of two linked members of MT I genes (MT IF and MT IG) and the MT IIA gene was studied in response to heavy metals and glucocorticoid hormone in human hepatoblastoma (Hep G2) and lymphoblastoid (WI-L2) cell lines. The MT IF and MT IG genes are expressed in Hep G2 cells but not in WI-L2 cells. The MT IIA gene is expressed in both cell lines in response to heavy metals but is induced by glucocorticoids only in Hep G2 cell line. These observations suggest that the MT I genes are expressed in a cell type specific manner and the induction of the MT IIA gene by glucocorticoids is cell type specific. The following evidence suggests that DNA methylation is involved in both phenomena: (i) azacytidine treatment of WI-L2 cells resulted in the expression of both MT I genes and glucocorticoid induction of MT IIA gene; (ii) transfection of WI-L2 cells with appropriate constructs containing MT promoters fused to CAT gene (MT-CAT) resulted in CAT expression in response to the inducers indicating the presence of active trans acting factors; (iii) analysis and comparison of the methylation pattern of these genes in both cell lines. In addition, azacytidine treatment of Hep G2 cells resulted in basal level expression of the MT IF and MT IG genes in this cell line, suggesting that DNA methylation also plays a role in control of basal level expression of MT genes. In conclusion, these results suggest that the expression of MT genes is controlled both by general methylation and site specific methylation, which results in cell type specific induction by the specific inducers. (Supported by AHFMR and MRC).

Metal Ion Homeostasis:
Molecular Biology and Chemistry

Z 109 THREE MAGNESIUM TRANSPORT SYSTEMS FROM SALMONELLA TYPHIMURIUM.

M.E. Maguire, M.D. Snavely, S.P. Hmiel and C.G. Miller, Depts. of Pharmacology and of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106

Three loci, designated corA, mgtA, and mgtB, are involved in Mg^{2+} transport in Salmonella typhimurium. Since Co^{2+} is a competitive inhibitor of Mg^{2+} influx and toxic to gram-negative bacteria, strains resistant to Co^{2+} were selected (Hmiel et al. (1986) J. Bacteriol. 168:1444-1450); such mutations were designated corA. Mg^{2+} influx is reduced in corA strains grown in high but not low Mg^{2+} concentrations while Co^{2+} uptake is abolished under either growth condition, implying the existence of an inducible/repressible component of Mg^{2+} uptake. A corA strain mutagenized and subjected to ampicillin selection for mutants requiring high Mg^{2+} concentrations for growth yielded strains with mutations at two new loci, mgtA and mgtB. Strains containing mutations at all three loci lack Mg^{2+} uptake under any growth conditions and require mM Mg^{2+} in the growth medium for survival. Plasmids carrying inserts uniquely complementing each locus were identified from a S. typhimurium chromosomal library; the chromosomal genes carried by the plasmids map at distinct loci. Subclones of each were prepared and each shown to code for only a single polypeptide with molecular weights of 41 kD, 37 kD and 102 kD for corA, mgtA, and mgtB, respectively. Because each of the three genes codes for a single polypeptide and because reintroduction of any one of the three genes restores Mg^{2+} transport and the ability to grow on low Mg^{2+} concentrations, these loci likely represent three distinct Mg^{2+} transport systems. Finally, the expression of mgtB is repressible by increasing concentrations of Mg^{2+} in the growth medium. This regulation is mediated by an additional locus, designated mgr for Mg^{2+} regulation. (NIH GM26340)

Z 110 SHEEP METALLOTHIONEIN GENES AND COPPER HOMEOSTASIS,

Julian F.B. Mercer, M. Gregory Peterson, Jennifer Smith, John Mc C. Howell* and Paul Gill*, Murdoch Institute, Royal Children's Hospital, Parkville, Vic. Australia, *Murdoch University, Perth, W.A, Australia.

Sheep are very susceptible to copper toxicosis due to reduced biliary excretion. In copper poisoned sheep much of the excess copper is thought to be in the form of polymerized metallothionein.

In order to study the role of metallothioneins in copper metabolism in the sheep, we have isolated a cluster of metallothionein genes from a sheep cosmid library. Two overlapping cosmid clones containing one MT-II gene, three complete MT-I genes (MT-Ia, Ib and Ic) and a truncated MT-I pseudogene were isolated. We have obtained the complete sequence of these genes and they resemble the mouse and human metallothionein genes in their overall structure. The regulation of this metallothionein gene cluster in sheep fibroblasts has been examined. The relative levels of expression following metal induction was MT-Ia > MT-II > MT-Ic >> MT-Ib. Only the MT-II gene responded to dexamethasone. The MT-Ia gene has a very low level of expression in the absence of inducers. In sheep liver the same relative level of expression was observed, but the absolute amount of mRNA was unexpectedly high. The levels of MT mRNA in copper poisoned sheep was also examined and surprisingly no correlation with copper levels in the liver was found, however, there was a weak but significant correlation with zinc. The ontogeny of MT mRNA in the sheep liver was determined, at the earliest age examined (29 days gestation), very high concentrations on MT mRNA were found (800 mol/pg RNA of MT-Ia) ; zinc concentrations were also high (3,000 µg/g dry wt). At all the ages examined and in various tisues of the sheep the same relative expression of the genes was observed. We are currently assessing whether the unusual copper metabolism in the sheep is related to the high levels of MT mRNA found in the liver.

Z 111 STUDIES ON UBIQUITIN CARBOXY-EXTENSION PROTEINS.

Brett P. Monia, David J. Ecker, Tauseef R. Butt and Stanley T. Crooke. Department Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104 and Department Molecular Pharmacology, Smith, Kline and French Labs, King of Prussia, PA 19406-0939.

Ubiquitin is a 76 amino acid protein believed to be essential for ATP-dependent, non-lysosomal intracellular protein degradation. It is found in eukaryotic cells either free or covalently joined to a variety of cytoplasmic and nuclear proteins. Ubiquitin, encoded in the genome as a multigene family, is synthesized either as polyubiquitin (3-9 tandem repeats of ubiquitin) or fused to sequences encoding a carboxy-terminus extension of 52-80 amino acids. These ubiquitin carboxy-extension proteins (CEPs) are strikingly similar to nucleic acid binding regulatory proteins in that they are highly basic (30% lys and arg) and contain amino acid sequence homology with the zinc-finger domains of transcription factors IIIA (Xenopus) and ADRI (Saccharomyces). We have initiated studies on the structure and function of CEPs in eukaryotes by expressing them in bacteria, yeast and mammalian cell lines. When expressed in eukaryotic cells, we have found that the ubiquitin-CEP fusion protein is processed to free ubiquitin and CEP. We have purified the intact fusion protein as well as CEP and are currently investigating their structure, nucleic acid binding properties and expression pattern in mammalian cell lines.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 112 IRON (*SODB*) AND MANGANESE (*SODA*) SUPEROXIDE DISMUTASE REGULATION BY THE *FUR* LOCUS IN *ESCHERICHIA COLI*, Eric C. Niederhoffer, Cleo Naranjo, and James A. Fee, Division of Isotope and Nuclear Chemistry, Los Alamos National Laboratory, Los Alamos, NM 87545.

Regulation of ferric ion uptake is mediated by the FUR protein (*fur*) in *E. coli*. Mutant *fur*$^-$ strains manifest the lack of repressor protein with overproduction of siderophores, catecholates, hydroxamates, and several inner and outer membrane proteins responsible for iron transport. FUR probably binds to a specific section, denoted the "iron box" (GATAATGATAATCATTACT), in the promoter regions of DNA coding for iron uptake. The Fe box is also found in the manganese superoxide dismutase (*sodA*) promoter, overlapping with the DNA dependent RNA polymerase -35 binding region. Therefore, if *sodA* is repressed by FUR, MnSD would be constitutive in *fur*$^-$ mutants. Surprisingly, the *fur*$^-$ strain AB4020 expresses no MnSD under anaerobic conditions but has high levels of MnSD and no FeSD under O_2 or PQ^{2+} induction. *E. coli* AB402 (*fur*$^+$) expresses normal levels of *sodA* (MnSD) and *sodB* (FeSD) gene products. FeSD is expressed at low levels under anaerobic conditions and is induced to greater levels in the presence of 1mM Fe^{2+}. In *E. coli* AB402 *fur*$^+$ and AB4020 *fur*$^-$ which have been transformed with the high copy number plasmid pHS1-4 (*sodB*), FeSD is expressed; however, the level of expression of *sodB* is less in *fur*$^-$ than in the parent strain under all growth conditions. A reciprocal relationship between the levels of FeSD and MnSD is observed in these mutants. These results suggest that *sodB* is regulated under low Fe^{2+} conditions by a *fur* mediated repressor protein different from the FUR protein. (Supported by DOE/OHER and NIH GM21519. We thank Prof. J. Neilands for the mutant strains.)

Z 113 TUMOR METALLOTHIONEIN(MT):INTERSECTION OF ZN METABOLISM AND REACTIONS OF CIS-DICHLORODIAMMINE PT(II) (DDP). A. Pattanaik, Pu Chen, S. Krezoski, A. J. Kraker, J. Garvey, C. F. Shaw III, and D. H. Petering, University of Wisconsin-Milwaukee, Milwaukee, WI 53201

Ehrlich cells contain a readily measurable level of Zn-Mt in the absence of induction by metals. When cells are placed in a Chelex-100-treated medium (t=0 hrs), which limits extracellular Zn, they lose Zn specifically from Zn-Mt at least 4 times faster ($t_{½}$=1 hr) than 35-S-Mt protein degrades. When complete medium was added back to cells at t=4 hrs, there was a two hr lag, followed by rapid reestablishment of the control level of Zn in Zn-Mt. These results suggest that Mt is actively involved in Zn metabolism. The findings may have general applicability to tumors, for substantial Mt-like metal binding protein has been found in each of 12 neoplasms surveyed, including, examples of melanoma(B16/BL6/ADR), fibrosarcoma(KHT/11B), colon adenocarcinoma(11A, 16C, 36C, C26 clone 10), mammary carcinoma-(M25), P-388 leukemia, and S-180. Mt is also a prominent site of binding of DDP in Ehrlich cells. DDP reacts with Zn-Mt with a rate constant of 2×10^{-4} sec^{-1}, independent of the ionic strength of the medium. These results suggest that Mt may be an important reaction site for DDP in many tumors (Supported by NIH ES-04026 and CA-22184).

Z 114 REPRESSION AND METAL-RESPONSIVE ACTIVATION OF TRANSCRIPTION BY THE MerR PROTEIN. Diana M. Ralston, Mark L. Chael, Betsy Frantz, Myung Shin, Jeffrey G. Wright and Thomas V. O'Halloran. Dept. of Chemistry and BMBCB, Northwestern University, Evanston, IL., 60208.

We have shown by *in vitro* analysis that purified MerR protein autoregulates its own expression and is converted from a repressor to an activator of mercury resistance gene transcription in the presence of nanomolar concentrations of mercuric ion. By run-off transcription, we have shown that MerR protein and mercuric ion are sufficient for regulation of transcription by E. coli RNA polymerase of the bacterial *mer* operon. The possibility that MerR functions as an alternate sigma factor to provide *mer* promoter specificity to RNA polymerase was rejected, since reconstitution experiments with core RNA polymerase plus either MerR protein or sigma70 demonstrated the necessity of sigma70 for transcription. In addition, we have determined that activation of transcription by MerR protein is extremely specific and sensitive to Hg(II) ion at concentrations as low as 0.05μM. This concentration is comparable to the level which induces mercury resistance *in vivo*.

Z 115 ISOLATION, STRUCTURE AND CHARACTERIZATION OF A NEW ZEALAND RABBIT METALLOTHIONEIN-I GENE, Y.-C. Tam, A. Chopra, M. Hassan and J.-P. Thirion, Department of microbiology, Faculty of medicine, CHUS, Sherbrooke, Quebec, Canada J1H 5N4.
Screening of a rabbit genomic lambda library with mouse metallothionein-I cDNA resulted in the isolation of a 4,400 base-pair DNA fragment containing a rabbit MTI gene. The rabbit MTI gene shows similar features with the mouse, human, sheep and rat MT genes: two introns interrupt three exons at the same amino acid positions. Exon 1 of the gene encodes the 5' untranslated region and amino acids 1-9 1/3; exon 2 encodes amino acids 9 2/3- 30 1/3; exon 3 encodes amino acids 31 2/3-61 and the 3' untranslated region. The introns, the 3' flanking region and the 5' flanking region of the gene show typical structures of a functional eukaryotic gene. These include splicing signals, polyadenylation signal, transcription (CAP) site, TATA box and CCAAT box. Comparison of the promoter sequence of this gene with the promoters of other metallothionein genes identified a number of oligonucleotide sequences that are involved in basal level transcription as well as sequences which are recognized by trans acting proteins involved in the induction of the gene by heavy metals, glucocorticoids and alpha interferon. Binding sites for the transcription regulatory factors SP1, AP1, AP2 and AP3 were identifed in the rabbit MTI promoter. Two other conserved sequences, a region of diad symmetry and 4 regions of potential Z-DNA structure formation are also present in the promoter of the rabbit MTI gene. The functions of these sequences are unknown. The fact that these sequences are also detected in most other mammalian metallothionein promoters suggests that they are recognized by trans acting factors not yet isolated. The promoter region of the sequenced rabbit MTI gene, with its numerous potential regulatory sequences represents one of the more complete and extensive promoters isolated so far.

Z 116 PROCEDURES FOR ENHANCING THE UTILITY OF THE METALLOTHIONEIN PROMOTER FOR THE REGULATED EXPRESSION OF DOWNSTREAM OPEN READING FRAMES, Robert E. Thach, William E. Walden, Susan Daniels-McQueen and Lisa L. Smith, Washington University, St. Louis, MO 63130.
Procedures which enhance the inducibility of the mouse metallothionein I (mMT-I) transcriptional promoter in mouse C127 cells stably transformed by bovine papilloma virus have been investigated. These include: (i) induction with Zn^{2+} at low serum concentration, and (ii) use of a 'superinduction' protocol (presence of 1μ g/ml of cycloheximide during induction with Zn^{2+}, followed by 2 μ g/ml of actinomycin D). Use of procedure (i) alone gave a 15-to 20-fold induction of expression of a downstream open reading frame (ORF), which is comparable to the maximum inducibility achieved with mMT-I in other systems. Use of procedures (i) and (ii) in combination allowed to 50-fold induction. Three different reporter ORFs (rabbit ferritin L subunit, human chorionic gonadotropin α subunit, and human lutropin β subunit), in three different chromosomal contexts, responded to these procedures. The maximum rate of expression achieved was estimated at over 10^9 molecules per cell per day, which is 20% of the transformed cell's protein synthetic capacity. At these extremely high levels some of the induced products were cytotoxic. The utility of this system for the identification of cytopathic viral gene products is currently being investigated.

Z 118 COPPER-INDUCIBLE PROTEINS AND GENES IN MIMULUS GUTTATUS, David A. Thurman, David Salt, Andrew Sewall, Mark Thomas, Jo Rodrigues de Miranda and A. Brian Tomsett, Depts. of Botany and Genetics, University of Liverpool, Liverpool L69 3BX, U.K.
Mimulus guttatus is a dicotyledonous plant which has been extensively characterized for copper tolerance (M. R. Macnair, 1983, Heredity, 50, 283-293). We are undertaking a study of copper metabolism and its relationship to copper tolerance in both tolerant and non-tolerant populations of this species. On the addition of copper to the growth medium of copper-tolerant plants, a series of copper-binding components are synthesized which are absent in plants grown in the absence of copper, and are only present in trace amounts in non-tolerant plants. We are attempting to characterize these components by purification to homogeneity. By a series of chromatographic techniques, including HPLC, we suspect that at least two and perhaps as many as five individual copper-binding components are present. Amongst these we have preliminary evidence to indicate the presence of phytochelatins and in addition, a protein with a complex amino acid composition which could be a metallothionein. In parallel, we are attempting to clone copper-inducible genes from this organism in order that we can investigate the relationship of copper regulation of metabolism and copper tolerance. We have constructed a large cDNA library from polyA$^+$ RNA isolated from copper-induced roots of copper-tolerant plants. This library is being analysed by positive/negative screening using cDNA sequences from copper-induced and uninduced roots from copper-tolerant plants. We have isolated a series of putative copper-inducible cDNA clones which are currently being characterized.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 119 METAL RESISTANCE IN ASPERGILLUS NIDULANS,
A. Brian Tomsett, Katherine H. Hodges, Anne Phelan and David A. Thurman, Depts. of Genetics and Botany, University of Liverpool, Liverpool L69 3BX, U.K.
Aspergillus nidulans is a well-characterized ascomycete fungus suitable for genetical, biochemical and molecular biological analysis. We are undertaking a study of metal metabolism and metal tolerance in this organism for a range of metals. We have already described the isolation and characterization of cadmium-resistant mutants (R. N. Cooley, H. R. Haslock and A. B. Tomsett, 1986, Curr.Microbiol. 13, 265-268). Thusfar, the isolated mutants define two genes, cadA on chromosome IV and cadB on chromosome VI. The cadB locus is tightly linked to the methB gene which encodes an enzyme of methionine biosynthesis. We have also isolated a series of cobalt-resistant mutants. These can define two genes, cobA and cobB both of which map to chromosome VI but neither of which are tightly linked to cadB or methB. At present, we are investigating both cobA and cobB for defects in sulphur metabolism. Preliminary evidence suggests that mutants of sulphur metabolism are epistatic to cobalt resistance. Attempts to identify metal-binding proteins are also underway and we have preliminary evidence for a copper-binding protein.

Z 120 CHARACTERIZATION OF A REPRESSOR OF FERRITIN mRNA TRANSLATION FROM RABBIT RETICULOCYTES.
W.E. Walden[1], L. Gaffield[1], P.H. Brown[2], S.D. McQueen[2], D. Bielser[2], and R.E. Thach[2]. [1]Univ. of Illinois at Chicago, Chicago, IL 60612 and [2]Washington Univ., St. Louis, MO 63130.
The regulation of ferritin synthesis in response to iron is one of the clearest examples of specific translational repression in eukaryotes. To examine this phenomenon in vitro, poly A+ mRNA was translated in the wheat germ and the rabbit reticulocyte lysates and newly synthesized ferritin was detected by immune precipitation. Ferritin is one of the major proteins synthesized in a wheat germ lysate programed with this mRNA. In contrast, ferritin synthesis is barely detectable in reticulocyte lysates. This repression of ferritin mRNA translation can be transferred to the wheat germ lysate by adding reticulocyte S100. We have partially purified this activity from rabbit reticulocyte S100 using ion exchange and gel filtration chromatography. Repressor activity elutes from gel filtration columns with an apparent M_r of approximately 120-150. When added to a wheat germ lysate programed with poly A+ RNA from rabbit liver or from mouse A20 cells, this fraction represses ferritin synthesis by more than 95% in a specific and dose dependent manner; a variety of control mRNAs are not affected. Experiments to further characterize this repressor are in progress. (Supported by NIH, NSF and a UIC, CRB institutional award).

Z 121 CT/AG RICH REPETITIVE SEQUENCES ARE CONTAINED WITHIN A HUMAN MT I LIKE GENE LOCUS
Janis M. Walker and L. Gedamu*. Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada.
Human metallothioneins (MTs) are low molecular weight, cysteine rich proteins encoded by a multi-gene family consisting of approximately 14 members. The functional MT genes are composed of 3 exons interrupted by 2 introns. The protein consists of the beta domain, represented by exons 1 and 2 and the alpha domain, being exon 3. We have isolated and characterized a lambda clone containing a MT I-like sequence. From sequence analysis and comparison with other human MT genes, our data indicates the following: (i) a portion of the beta domain of the protein has been drastically altered (11 of the 21 amino acids derived from the coding region of exon 2 have been changed) however all of the 7 cysteine residues have been conserved; (ii) of the 30 amino acids comprising the alpha domain of the protein (represented by the coding region of exon 3) only 4 amino acid changes have been found, while the 11 cysteine residues have been conserved; (iii) repetitive sequences of the type $(CTTT)_{16}(CT)_{12}(CTTT)_2(CCTT)_7$ followed by a GA rich region have been located within the sequence upstream of exon 2, as well as in the 5' flanking region; (iv) the sequences representing exon 1 and the regulatory region of this gene have not yet been identified. As a result of the mutations in the coding region, it is unlikely the protein will be functional. The function of these CT/AG rich repetitive sequences is not known, although they may be involved in the mutation and inactivation of this gene during evolution. (Supported by MRC and AHFMR).

Z 122 THE **Mto** METALLOTHIONEIN GENE OF DROSOPHILA MELANOGASTER,
Maurice Wegnez, Raja Mokdad, Agnès Cadic-Jacquier and Philippe Silar, Université Paris XI, Bâtiment 445, 91405 Orsay Cedex, France.

Two very distinctive genes characterize the metallothionein system of Drosophila melanogaster. The gene **Mtn**, located in region 85E10-15, codes for a protein of 40 amino acids (Maroni et al., Genetics **112**: 493-504, 1986). We have cloned a cDNA from a cadmium resistant cell line which corresponds to a 43-amino acid metallothionein (**Mto**). The **Mtn** and **Mto** protein sequences are only 26% isologous (Mokdad et al., PNAS **84**: 2658-2662, 1987). We have further characterized the **Mto** gene. This gene is located, according to in situ hybridization, in region 92E (on the right arm of chromosome 3). It is interrupted, as found for **Mtn**, by a single intron. Interestingly, both introns are located at the same position when the sequences are aligned as proposed in our PNAS paper. This strongly suggests that the two genes are homologous and have greatly derived since a very ancient duplication event. Induction of both genes by metal ions (zinc, copper and cadmium) was studied in adult flies and culture cells.

Z 123 STRUCTURE OF THE RAINBOW TROUT METALLOTHIONEIN-B GENE: DIFFERENTIAL EXPRESSION IN RESPONSE TO HEAVY METAL INDUCERS, M. Zafarullah and L. Gedamu, Dept. of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4.

The trout metallothionein (MT) gene family is comprised of two members. To study regulation of these genes we have isolated one member and designated it tMT-B. The gene codes for a 60 amino acid protein and its overall structure is similar to mammalian MT genes. The three exons are interrupted by two introns of 108 and 635 nucleotides. The 5' flanking region has the following features: 1) a consensus TATAAA sequence, 2) two regions strongly homologous with the metal responsive elements (MRE) of mammalian MT genes, 3) in contrast with mammalian MT 5' flanking regions 63% AT richness, 4) lack of consensus Sp1 binding sites. Following heavy metal induction the gene is expressed at high levels in kidney, spleen and gills and at low levels in the liver. In the rainbow trout hepatoma (RTH) cell line, the gene responded well to Zn, moderately to Cd and not at all to Cu. Together these data indicate differential regulation of this gene by these metals in different cell types. The chinook salmon embryonic (CHSE) cell line synthesized tMT-B mRNA only after prior treatment with 5-azacytidine followed by exposure to metals suggesting regulation of this gene by DNA methylation. Fusion of a DNA fragment encompassing -600 to +8 (which contains the TATA and MREs) with the CAT gene and its transfection into the RTH cell line showed that the cis-acting elements necessary for regulation of CAT by metals are present within this fragment. (Supported by AHFMR, NSERC and MRC).

Structure and Chemistry of Metalloproteins

Z 200 **STRUCTURE AND FUNCTION OF PHYTOFERRITINS**, Jean-Pierre Laulhère, Anne-Marie Lescure and Jean-François Briat, Laboratoire de Biologie Moléculaire Végétale, CNRS UA 1178, Université de Grenoble 1, BP 68, 38402 Saint Martin d'Hères, France.

Ferritins from seeds of pea, soyabean and maize have been purified as an oligomer build up from two polypeptides of respectively 28 KDa and 26.5 KDa. The molecular mass of the native pea seed protein is of 540000. Pea and maize seed ferritins have been compared. Their amino acid compositions are very similar. Four isoforms of the 28 KDa polypeptide from pea have been observed instead of a unique polypeptide for the maize one. No isoforms have been detected for the 26.5 KDa polypeptide. Rabbit antibodies raised against pea seed ferritin have been used: ferritins of the three plants analysed share immunological determinants. It is also shown that ferritins are not uniformly distributed among different pea organs of iron unloaded 30 days old plants. The protein is more abundant in flowers than in fruits and roots and not detectable in leaves.
In vitro iron release and uptake by pea seed ferritin has been studied. It is shown that ascorbate, a reducing agent present within chloroplasts, is able to mobilize iron by reduction at a much faster rate than a release by chelation of ferric iron. The reduction is accompanied by partial reoxydation into the mineral core as well as a release of ferrous iron which accumulates in the medium. Reduction appears to be a necessary step for the release as well as for the uptake of iron inside ferritin molecules. No incorporation of iron inside the protein occurs in the absence of ferrous iron. A reduction of ferritin iron occurs in the absence of ascorbate.
Phytoferritin does not appear only as a ferric chelate among a series of stronger or weaker ferric complexes; it appears to interact with ferrous ions as an oxydant and with ferric ions as a reducer. Therefore, the uptake and release of iron ferritin depend upon the redox potential of the protein more than of its stability as a ligand for ferric or ferrous iron.

Z 201 STRUCTURE AND FUNCTION OF COPPER METALLOTHIONEIN FROM THE AMERICAN LOBSTER *Homarus americanus*. Marius Brouwer and Dennis Winge, Duke University Marine Lab, Beaufort, NC and University of Utah Medical Center, Salt Lake City, UT.

Hemocyanins are multisubunit proteins, which contain binuclear-Cu(I) centers that can combine with oxygen or carbon monoxide. The apo-protein can be reconstituted *in vitro* with Cu(I), but not with Cu(II). Recent studies suggest that Cu(I)-metallothionein (Cu-MT) may act *in vivo* as a Cu(I) donor to apohemocyanin (Engel and Brouwer ,1987, Biol. Bull. 173, 239). To further explore this putative function of Cu-MT , we have isolated a copper protein from the digestive gland of the American lobster, which was identified as a MT based on its amino acid sequence. Copper transfer, from this Cu-MT to lobster apohemocyanin, and formation of binuclear-copper sites that can bind CO and oxygen were followed by fluorescence and electronic absorption spectroscopy. These studies showed that formation of CO-binding sites precedes the formation of oxygen-binding sites. Since the binding of oxygen, which bridges between two coppers (Cu-O-O-Cu) is subject to greater stereochemical constraints than the binding of CO, which binds to only one copper (Cu...Cu-CO), we conclude that Cu(I) transfer from MT to apohemocyanin initially results in the formation of distorted binuclear-copper sites which subsequently slowly return to their native stereochemical configuration.

Z 202 EXPRESSION OF A CHINESE HAMSTER METALLOTHIONEIN CODING SEQUENCE AND ITS VARIANTS IN *SACCHAROMYCES CEREVISIAE*. Mary Cismowski, Mark Chernaik, In-Koo Rhee, and P.C. Huang, The Johns Hopkins University, Baltimore, MD 21205.

Metallothionein (MT) is a low molecular weight cysteine-rich metal binding protein found in all mammalian species studied to date, as well as in the yeast *S. cerevisiae*. Efficient yeast expression vectors have been developed (Ecker, *et al*., JBC 261, 16895) which express foreign coding sequences from either a copper inducible (cupl) or constitutive (TDH) promoter; detection of exogenous MT expression is facilitated by the use of a yeast MT^- ($cupl^-$) strain. Wild type and mutated CHO MT2 cDNA sequences (Pine, *et al*., DNA 4, 115) were introduced into this yeast expression system. All MT constructs studied so far, when introduced into yeast $cupl^-$ cells, confer increased resistance to the toxic effects of exogenously added metals. Wild type CHO Zn-MT2, as well as CHO Zn-MT2 mutants with single or multiple cys--tyr amino acid changes, have been purified and characterized from this yeast system. This work was supported by NIH grant R01GM32606.

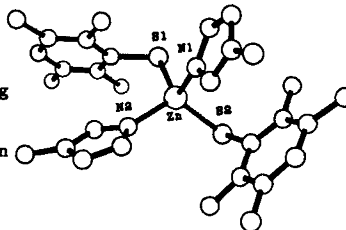
Z 203 AN EXAFS STUDY OF THE COPPER ACCUMULATED BY A COPPER-RESISTANT MAMMALIAN CELL LINE, Jonathan H. Freedman, J. Peisach, L. Powers, Albert Einstein College of Medicine of Yeshiva Univ., Bronx, NY, 10461, and AT&T Bell Laboratories, Murray Hill, NJ, 07974.

X-ray absorption spectroscopy has been applied to the *in vivo* examination of a Morris hepatoma cell line that accumulates copper and is resistant to metal toxicity (Freedman, J.H., Weiner, R.J., and Peisach, J. (1986) *J. Biol. Chem.* 261: 11840-11848.) Analysis of the K-absorption edge suggests that the accumulated copper is univalent and is complexed in a distorted tetrahedral geometry. Analysis of the extended X-ray absorption fine structure data indicates that copper is coordinated by four sulfur atoms at a distance of 2.28 ±.02 A. In addition, a copper atom is located in the outer coordination shell 3.69 ±0.05 A from the absorbing metal. The structural arrangement of the copper and sulfur in these cells is similar to that observed in a purified copper metallothionein (Freedman, J.H., Powers, L., and Peisach, J. (1986) *Biochemistry* 25: 2342-2349.)

Z 204 **PROTEINS WITH THE POTENTIAL TO CONFER ALUMINUM RESISTANCE ON PLANTS**
Richard C. Gardner and Joanna J. Putterill, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand
Aluminum toxicity is an extremely important problem in agriculture, affecting crop yields on up to 40% of the worlds arable soils. Metal chelation is the predominant defence mechanism in both plants and animals for metals such as Cd and Zn. We have decided to attempt to develop a protein which can chelate free aluminum as a strategy to protect plants against the toxic effects of the metal. Work will be described in which the ability of various proteins to bind aluminum is assessed. We have used equilibrium dialysis, and two additional assays which measure the ability of various test compounds to compete with calmodulin for aluminum binding. Aluminum binding to calmodulin can be assessed by monitoring ANS fluorescence or by measuring its stimulation of phosphodiesterase activity. We have included test compounds in both of these assays in order to assess their capacity to reduce the effects of aluminum on calmodulin. The order of the test compounds in their ability to bind aluminum in these three assays was: polyaspartic acid/polyglutamic acid/transferrin > citric acid/calmodulin > bovine serum albumin >> metallothioneins/enkephalins. We are currently attempting to express some of these aluminum-binding proteins in plant cells in order to ascertain whether they have a protective effect on the cells under conditions of high aluminum.

Z 205 **ZINC, COBALT AND CADMIUM THIOLATE COMPLEXES: MODELS FOR THE $Zn(S\text{-}CYS)_x$ CENTERS IN DNA BINDING PROTIENS AND METALLOENZYMES,**
Stephen Koch, Douglas T. Corwin, Jr., and Eric Gruff, Department of Chemistry, SUNY Stony Brook, Stony Brook, NY 11794.

$Zn(S\text{-}cys)_x$ (x = 1-4) coordination centers have been proposed or established in a wide range of metalloproteins including the recently proposed zinc finger proteins. A series of $[Zn(SR)_x(imidazole)_{4-x}]$ complexes have been synthesized to serve as structural models for the protein centers. Analogous cobalt(II) and cadmium(II) complexes have been prepared to serve as spectroscopic models for metal-substituted zinc centers. Electronic spectroscopy and ^1H NMR has been used to study the cobalt(II) complexes while the cadmium complexes have been studied using ^{113}Cd NMR. Structural and spectroscopic criterion have been established which should enable the identification of the coordination number and geometry of zinc-cysteine centers in metalloprotein and for the determination of the number of cysteine residues coordinated to these centers.



Z 206 CU TRANSFER AND XAS STUDIES OF BLUE COPPER SITES,
Angela M. Schmidt, Kelley A. Burns, David R. McMillin and James E. Penner-Hahn, Purdue University, West Lafayette, IN 47907

The published crystal structure of Hg-plastocyanin shows that the basic structure of the metal binding site is similar to that of the native protein although expanded to accommodate the larger metal ion. We have examined the Hg EXAFS signals from plastocyanin, azurin, stellacyanin and <u>Rhus</u> laccase derivatives containing Hg(II), and we find that mercury is bound in the blue copper site of each. Moreover, each site contains 2N(His) and 1S(Cys) donors in the first coordination shell. This is the first direct probe of the ligand set of the type 1 site in <u>Rhus</u> laccase. From the second shell data the type 1 site in stellacyanin seems to be more like that in azurin than plastocyanin. Some copper transfer experiments will also be described. In the presence of ascorbate laccase can transfer copper to apoazurin and apostellacyanin. However, <u>Rhus</u> laccase appears to be heterogeneous in solution, and copper donation may stem from modified protein.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 207 Distinct mechanism of cadmium and copper resistance in Torulopsis glabrata, R.K. Mehra, E.B. Tarbet, W.R. Gray and D.R. Winge, Medicine and Biochemistry, University of Utah Medical Center, Salt Lake City, Utah 84132
All the animal species studied to date respond to heavy metal toxicity by synthesizing a cysteine-rich polypeptide, metallothionein. However, a γ-glutamyl containing cysteine-rich peptide is synthesized by plants upon exposure to heavy metals. Species within Protista and Fungi are known to synthesize either γ-glutamyl peptide or cysteine-rich polypeptide similar to MT but not both of them. Torulopsis(Candida) glabrata is highly resistant to both copper and cadmium; .8mM and >1mM concentrations of copper and cadmium, respectively, are required to cause a 50% reduction in the growth of cells in a minimal medium. A combination of gel filtration and ion-exchange procedures was utilized to purify metal-binding species from the cells grown in copper or cadmium salts. The analysis of amino acid composition and partial sequence of copper-binding components showed that the metal was bound to two distinct cysteine-rich polypeptides that differ from each other in their primary structure. The physico-chemical characterization of the purified Cd-binding species from T. glabrata cells grown in 1 mM $CdSO_4$ showed that the metal was exclusively bound to γ-glutamyl containing cysteine-rich polypeptides previously isolated from many plants and S. pombe.

Z 208 IRON-MOLYBDENUM COFACTOR OF AZOTOBACTER VINELANDII NITROGENASE, William E. Newton, Stephen F. Gheller and Benjamin J. Feldman, Western Regional Research Center, USDA/ARS, Albany, CA 94710; Franklin A. Schultz, Department of Chemistry, Indiana University - Purdue University at Indianapolis, Indianapolis, IN 46223; and Britt Hedman, Patrick Frank and Keith O. Hodgson, Department of Chemistry, Stanford University, Stanford, CA 94305.
Iron-molybdenum cofactor (FeMoco) is the Fe-Mo-S-O-containing cluster of nitrogenase, which constitutes the putative active site of biological dinitrogen reduction to form ammonia. FeMoco is extracted from the MoFe protein of the nitrogenase complex into N-methylformamide, where it exists as a low-molecular-weight entity of composition $MoFe_{6-8}S_{8-10}O_{>2}$. Direct electron exchange with electrodes can convert extracted FeMoco among its oxidized, semi-reduced and fully-reduced oxidation states. FeMoco has been studied in these states by electrochemical, EXAFS and EPR methods. The results indicate that FeMoco exists in more than one form in its oxidized and semi-reduced states and that the fully reduced state exhibits electrocatalytic activity. Relationships between these forms and states and the electron-transfer reactivity of FeMoco are apparent. The significance of these observations with respect to biological nitrogen fixation will be presented.

Z 209 **THE BIOCHEMICAL CHARACTERIZATION OF A METALLOTHIONEIN-LIKE PROTEIN FROM BOVINE HIPPOCAMPUS, V. K. Paliwal, T. Takahashi, and M. Ebadi. Dept. of Pharmacol., Univ. of Neb. Coll. of Med., 42nd St. and Dewey Ave., Omaha, NE 68105**
Mammalian hippocampi not only contain high concentrations of dithizone chelatable zinc, but also exhibit regional variation in this essential element, with concentrations being highest in the hilar region and lowest in the fimbria. For example, the concentration of zinc in the mossy fiber axons has been estimated to approach 300-350 µM. In an attempt to investigate further the dynamic metabolism of zinc, we have searched for and have identified a metallothionein-like protein in bovine hippocampus with the following properties: similar to the zinc-induced hepatic metallothionein (hep-MT), the hippocampal metallothionein-like protein exhibits an elution volume (Ve/Vo) of 2.0 on gel filtration chromatography, and produces two isoforms, which on a reverse phase high performance liquid chromatography (HPLC) show retention times of 16.72 min. (hep-MTI produces 16.53 min.) and of 17.94 min. (hep-MTII produces 18.45 min.), respectively. The hippocampal metallothionein isoform II contains a cysteine to zinc ratio of 2.8 to 1.0, and, as shown by studies involving UV spectral analysis, apparently lacks aromatic amino acids, but possesses metallomercaptide bonds. Investigations to determine the regional CNS localization, as well as the subhippocampal distribution of this metallothionein-like protein, are in progress. **(Supported in part by a grant from USPHS ES-03949.)**

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 210 SULFIDE STABILIZATION OF THE CADMIUM-γ-GLUTAMYL PEPTIDE COMPLEX AND EXTRACELLULAR PRECIPITATION OF HEAVY METALS. R.N. Reese, A.K. Hughes and D.R. Winge, Medicine and Biochemistry, University of Utah Medical Center, Salt Lake City, Utah 84132

Sulfide production in response to heavy-metals appears to be a cellular mechanism by which many plants, fungi, protists and bacteria can increase their metal tolerance. In plant cells and fission yeast, sulfide acts to enhance the effectiveness of the γ-glutamyl-peptide system of cadmium detoxification and may act in a secondary role to increase metal tolerance through precipitation of Cd(II) extracellularly as CdS. Native Cd-γ-glutamyl-peptide complexes contain acid labile sulfur in the metal-thiolate cluster. Two forms of the Cd-peptide complex have been described, differing primarily in their sulfide content. High sulfide forms of the metallopeptide complex ($\geq$ 1 mol sulfide per mol peptide) showed a sulfide-dependent electronic transition in the ultraviolet, with an absorption peak at 318 nm. Incorporation of sulfide into the Cd-peptide complex imparted greater thermodynamic stability to the complex, increased the Stokes radius and enhanced the cadmium binding capacity in comparison to that of the low sulfide forms. Addition of sulfide ions to the low sulfide metallopeptide complex caused its conversion to the high sulfide form. Increased sulfide production and incorporation into γ-glutamyl-peptide complexes generated in response to copper have not been observed, but may play a minor role in sequestration of toxic levels of zinc.

Z 211 FERRITIN HETEROGENEITY CAN BE DUE TO BINDING BY NON-FERRITIN PROTEINS: ALPHA-2-MACROGLOBULIN (A2M), Paolo Santambrogio and William H. Massover, UMDNJ-New Jersey Medical School, Newark, NJ 07103.

Heterogeneity of molecular dimers in rabbit liver ferritin is revealed by the presence of two discrete beta bands with pore-gradient PAGE; in addition to H and L subunits (SDS-PAGE), an 170kDa polypeptide is present only in the beta band of larger dimers (1987 Biochem. Biophys. Res. Comm. 148:1363-1369). We have investigated the nature of this polypeptide. Double immunodiffusion with anti-rabbit-A2M against rabbit serum, unfractionated rabbit liver ferritin, and the purified band of larger dimers causes a precipitation reaction showing complete identity; no immunoprecipitate is formed against ferritin in the bands of molecular monomers or smaller dimers. The anti-A2M immunoprecipitate formed with ferritin, but not that with serum, stains positively for iron content (Perl's reaction); thus, anti-A2M causes the precipitation of ferritin, but only that within the larger dimers. Immunoblotting with transfers from PAGE and SDS-PAGE shows that anti-rabbit-A2M antibodies react strongly only with the larger dimers and the 170kDa polypeptide; this indicates that the larger dimers contain A2M, the 170kDa polypeptide is A2M, and these antibodies do not cross-react with H or L types of ferritin subunits. Electron microscopy of pure alpha band monomers and of each beta band shows that all contain many ferritin molecules; the larger dimers uniquely also have many "non-ferritin" proteins with the highly characteristic ultrastructure of A2M. Thus, A2M binds some rabbit liver ferritin, and this binding causes a molecular heterogeneity of ferritin. (Supported by grant DK34340.)

Z 212 SPECTROSCOPIC STUDIES OF HAFNIUM-TRANSFERRIN COMPLEXES: STRUCTURE OF THE METAL-BINDING SITES.

D.M.Taylor, W.-G.Thies, Kernforschungszentrum Karlsruhe, Institut für Genetik und für Toxikologie von Spaltstoffen, Postfach 3640, D 7500 Karlsruhe, F.R.G. H.Appel, M.Neu-Müller, and H.W.Weber, Universität Karlsruhe, Institut für Experimentelle Kernphysik, Postfach 3640, D 7500 Karlsruhe, F.R.G.

The structure of the metal-binding sites in ^{181}Hf-labeled transferrin was studied using the Time Differential Perturbed Angular Correlation (TDPAC-) Method. This technique is highly sensitive to the local environment of the metal-ions. Hafnium-transferrin complexes were prepared in serum and in different buffer systems. Two well-defined hafnium-binding configurations were observed. It could be shown that the presence of phosphate in the buffer medium is essential for a specific metal-binding. The distribution of the hafnium-ions between the binding configurations is strongly affected by the temperature and the pH of the samples. Difference Ultraviolet Spectroscopy on iron- and hafnium-transferrin complexes was used to compare the number of tyrosines coordinated to the metal at the binding sites.

Metal Ion Homeostasis: *Molecular Biology and Chemistry*

Z 213 A GEOMETRICAL ANALYSIS OF METAL-BINDING SITES IN PROTEINS. Keith B. Ward, Linda I. Hannick, N. Pattabiraman and Bruce Gaber[a] *(Laboratory for the Structure of Matter, Code 6030,* [a]*Biomolecular Engineering, Code 6190, Naval Research Laboratory, Washington, DC 20375)*
In order to survey the spatial distribution of metal-binding ligands in proteins, we have geometrically analyzed the metal-binding sites of protein structures in the Brookhaven Protein Data Bank. Proteins that bind metals contain the following metal ligands: 1) oxygen atoms in carboxylic, carbonyl, hydroxyl and carbamide groups; 2) nitrogen atoms in amide, carbamide, imidazole, guanidinium and ammonium groups and 3) sulfur atoms in thiol and methionyl groups. In addition atoms in solvent molecules, ions in solution, other metal ions, substrates and prosthetic groups can serve as ligands for the bound metal. After extracting the coordinates of all atoms within 6 Å of the bound metal, we construct histograms indicating the number of oxygen, nitrogen and sulphur atoms as a function of distance from the metal. We classify the oxygen and nitrogen atoms into five types depending upon the atoms to which they are attached. To compare metal binding sites in different proteins, we define an orthogonal coordinate system in which the metal is at the origin, the x-axis is along the shortest atom-metal distance and the X-Y plane is defined by the metal and the two ligand atoms closest to the metal. Positions of atoms in the metal binding site are then expressed in spherical coordinates referred to this local system. A phi-theta plot is made for all atoms that are within the metal-ligand coordination distance (~3 Å), which easily allows us to compare and contrast the geometries around the metals. The histograms are useful in understanding electrostatic effects due to other nearby atoms that are not directly involved in coordination to the metal. Results obtained by applying this analysis to metallothionein and other metal-binding proteins will be presented. (Supported by the Office of Naval Research, contract N0001487WX22283.)

Z 214 COORDINATION CHEMISTRY OF METALLOTHIONEIN AND ITS SYNTHETICALLY PREPARED DOMAINS. Dean E. Wilcox, Timothy E. Elgren, Michael F. Reed, F. Jon Kull, Thomas Ciardelli, Department of Chemistry, Dartmouth College, Hanover, NH 03755.

The metal ion binding properties of metallothionein are intimately related to its physiological role. We have begun to employ vibrational spectroscopic methods to study the coordination chemistry of metallothionein and to probe the structure of metallothionein reconstituted with different metal ions. FT-IR, Raman and resonance Raman techniques have been used to monitor S-H and M-S stretching frequencies in our studies of metal ion interaction with the cysteine residues. Our studies have now been extended to the isolated metallothionein domains which we have successfully prepared using solid phase peptide synthesis. This methodology is now being used to modify the primary structure of the domains to investigate the contributions of individual residues to the metal binding properties of metallothionein.

Z 215 CORRELATION OF THE STRUCTURE OF HUMAN TRANSFERRIN WITH THE TRITIUM EXCHANGE KINETICS IN HISTIDINYL C(2)H's OF THE N-TERMINAL LOBE, Andre A. Valcour and Robert C. Woodworth, University of Vermont College of Medicine, Burlington, VT 05405.
The exchange of histidinyl C(2) protons with ^2H or ^3H in the medium is enhanced at elevated pH and temperature. In metalloproteins in which His sidechains serve as ligands to metal ions, this exchange is often inhibited or prevented altogether. Having found in proton magnetic resonance studies that the binding of iron by the N-terminal half-molecule of human transferrin (TF/2N) protects three titrable C(2)H against exchange with ^2H_2O, we sought to locate the relevant His residues within the primary structure. The iron-free protein was allowed to exchange with ^3H_2O for eight days at pH 8.2 and 42°. The TF/2N was saturated with iron, lyophilized, dissolved in H_2O and allowed to back-exchange for 10 days. The lyophilized residue was dissolved and dialyzed vs chelators to remove iron. Performic acid oxidation followed by tryptic digestion yielded a mixture of peptides, which were resolved by FPLC and HPLC into a series of tritiated peptides. Several of these have been characterized by amino acid analysis and have been located within the primary sequence. Analysis of the rates of ^3H back-exchange suggests that ^{119}His is highly protected and ^{249}His and ^{273}His are significantly protected in the iron complex. ^{207}His was not located to an isolable peptide, but appears to lie within the binding site region when placed within the known crystal structure of human lactoferrin (LTF). This substitution of His in TF for Asp in LTF may be a key to the different iron-binding affinities of TF and LTF. Supported by USPHS Grant DK21739.

Z 216 **FACTORS INVOLVED IN THE REGULATION OF THE HUMAN METALLOTHIONEIN IIA GENE**

Petra Skroch and Michael Karin
Dept. of Pharm. M-036, School of Medicine, UCSD, La Jolla, CA 92093

The human metallothionein gene cluster contains at least 12 distinct MT genes. Their expression is regulated at the transcriptional level by heavy metal ions, steroid hormones, interferon, interleukin I, serum factors and phorbol esters in a tissue specific manner. Up to now only one gene of the MT II isotype was detected whereas the MT I population is heterogeneous. In all human cell lines and primary tissues examined so far the MT IIA gene was expressed at much higher levels than the MT I genes. The MT IIA promotor region was analyzed in detail and found to contain no less than five distinct control elements, which can be functionally subdivided into two categories: basal and induced elements. There are several recognition sequences for factors affecting basal transcription, which include a TATA box, a GC box and at least two basal level enhancer sequences. Interspersed between these elements are sequences are sequences that confer metal inducibility, the so called metal responsive elements (MRE). The MREs of human MT genes share a highly conserved sequence which is also similar to MREs of other species, such as mouse or rat. All of the examined human MT genes contain more than one MRE, although their regulatory regions in general don't have the complexity of that of the MT IIA gene. Deletion of the MREs has no effect on the basal activity of the promotor but prevents further induction by Cd. The function of regulatory DNA sequences is mediated by interaction with cellular factors, that specifically bind to these sequences. Up to now three trans acting factors involved in the regulation of the MT IIA gene have been identified and purified: SP1 that binds the GC box, AP1 a distinct sequence within the proximal BLE and AP2 that binds to three sites in the proximal and distal BLEs. Another regulatory protein that binds to the MT IIA gene is the glucocorticoid hormone receptor. In vivo the MT IIA promotor can compete with the SV40 enhancer for one or more cellular factors. This competition is modulated by Cd, suggesting that a metal regulatory protein (MRP) might stabilize the binding of limiting factors such as AP1 or AP2 to the BLEs in the presence of heavy metal ions.

To characterize and isolate a trans acting factor that interacts with MRE sequences of the hMT IIA gene we performed gel retardation experiments. A DNA fragment containing a synthetic MRE sequence was incubated with HeLa whole cell extract or further purified fractions. Both caused a change in the mobility of part of the DNA molecules. The disappearance of certain complexes in the presence of competing MRE DNA demonstrated the specificity of the binding reaction. At the moment we are further characterizing and purifying this binding activity.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Detoxification and Disease States

Z 300 BACTERIAL REDUCTION OF SELENITE TO ELEMENTAL SELENIUM, Robert Blake II, Smriti Bardhan, Julius Jackson*, and Nathaniel Revis+, Meharry Medical College, Nashville, TN 37208, *Michigan State University, East Lansing, MI, 48824, +Oak Ridge Research Institute, Oak Ridge, TN, 37830

A Pseudomonas sp. isolated from mercury-contaminated soil at Oak Ridge, Tennessee could be adapted to grow in the presence of either mercuric ions (100 μM) or sodium selenite (50 mM). When grown aerobically in the presence of selenite, the organism affected the precipitation of the red, metallic form of elemental selenium. Washed cell suspensions of the bacteria were capable of reducing selenite only when organic nutrients such as glucose were included in the external medium. Cell suspensions of selenite-grown bacteria did not transform detectable amounts of sulfite, selenate, tellurite, or arsenite under the same experimental conditions. A quantitative colorimetric assay for free selenite was developed from the interaction of selenite with 2-mercaptobenzimidazole in 0.01 N sulfuric acid to form a 1:1 complex with a unique absorbance peak at 360 nm. The accuracy of this colorimetric assay was determined by parallel measurements using atomic absorption spectrometry. The organism harbors a 23 kb plasmid that appears to be essential for resistance to selenite. Preliminary efforts to identify a cell-free selenite reduction activity indicate that the activity may reside in a membrane fraction. This work was supported by the Department of Energy through Subcontract ORRI-85-4 from the Oak Ridge Research Institute.

Z 301 EFFECTS OF ZINC ADMINISTRATION ON CELLULAR ZINC AND IMMUNITY IN THE ELDERLY, John Bogden, James Oleske, Marvin Lavenhar, Elizabeth Munves, Francis Kemp, Kay Bruening, Kimberly Holding, Thomas Denny, Michael Guarino, and Bart Holland, UMD-New Jersey Medical School, Newark, NJ 07103-2757

Severe Zn deficiency in animals and humans produces impaired cellular immunity, and pharmacologic doses of Zn may enhance immune function even in the absence of Zn deficiency. The objective of this study was to determine the effects of Zn supplementation on Zn concentrations in circulating cells and on cellular immunity in the elderly. One hundred subjects, aged 60-89, were given a placebo, 15 mg Zn or 100 mg Zn daily for up to 12 months. All subjects also received a multivitamin/mineral supplement that contained no additional Zn. Blood samples were drawn prior to and at 3, 6, 12, and 16 months after beginning Zn supplementation. Plasma Zn increased significantly only in the 100 mg Zn treatment group, from 86±2 to 110±4, 111±5, and 109±4 μg/dl at 0, 3, 6, and 12 months respectively. Concentrations of Zn in erythrocytes, lymphocytes, neutrophils, and platelets were not significantly increased. Four months after stopping Zn administration, plasma Zn in the 100 mg Zn treatment group fell to 84±4 μg/dl. There was a progressive improvement in delayed dermal hypersensitivity and lymphocyte proliferative responses to a panel of mitogens and antigens, but these effects were not related to the Zn treatment group. The inability of Zn supplementation to enhance cellular immunity in most of the elderly subjects studied may be related to the absence of an increase in the zinc content of immunologically active cells despite significantly increased plasma zinc concentrations. (Supported by NIH grant AG04612.)

Z 302 METAL HOMEOSTASIS DURING THE MOLT CYCLE, David W. Engel, National Marine Fisheries Service, NOAA, Southeast Fisheries Center, Beaufort Laboratory and Marius Brouwer, Duke Marine Laboratory/Marine Biomedical Center, Beaufort, NC 28516.

One of the continuing enigmas of metallothionein research is the apparent lack of a clear correlation between metallothionein function and a specific physiological process. In our Laboratories we have demonstrated direct correlations between copper and zinc metabolism, the molt cycle of the blue crab, Callinectes sapidus, and metallothionein. We have shown that during the molt cycle there are significant losses of both metals at molt that are manifested as changes in both total metal concentrations in the digestive gland and hemolymph, and changes in the copper / zinc ratios associated with metallothionein in the digestive gland cytosol. In addition there also is a positive correlation between the amount of copper bound to metallothionein and the turnover of hemocyanin (i.e. a copper containing respiratory protein in crustaceans). Our data, therefore, suggests that during the critical stages of the molt cycle of growing blue crabs there is a direct involvement of metallothionein both in copper sequestration and excretion and in copper donation for hemocyanin synthesis and zinc metabolism.

Z 303 COMPARISON OF CADMIUM AND METAL BINDING PROTEIN KINETICS IN TWO SPECIES OF FISH, John M. Frazier, Sandra M. Baksi, Tracie E. Bunton and Anne Loftus, The Johns Hopkins University, Baltimore, MD 21205. Recent studies in this laboratory have investigated an unusual fish species, the white perch (Morone americana), which accumulates exceptionally high concentrations of hepatic copper under conditions of normal copper exposure. The mechanisms which account for the hepatic copper accumulation in this species are unknown, but may be related to genetically controlled molecular mechanisms at the hepatic level since a closely related fish species, the striped bass (Morone saxatilis), exhibits normal hepatic copper metabolism. One mechanism which could account for this effect is differences in the regulation of metal binding protein (MBP) synthesis. This study compared cadmium and MBP kinetics between the two species of fish following injection of cadmium. Cadmium was selected as a research probe since it is a known inducer of metallothionein in mammals and neither fish species contained measurable levels of hepatic cadmium. Both species accumulated similar levels of hepatic cadmium following repeated injections. However, significant differences were observed in the kinetics of cadmium association with MBP (less cadmium was bound to MBP in white perch compared to striped bass) and the behavior of the essential metal zinc (again lower levels of zinc associated with MBP in white perch). The high and variable levels of copper associated with MBP in white perch made it difficult to measure the level of induction of MBP in this species. Whether the differences observed are due to differences in the fundamental regulation of MBP or due to the interactions of high hepatic copper levels with cadmium induction of MBP synthesis must be resolved.

Z 304 STUDIES OF THE HLA CLASS I REGION IN HEREDITARY HEMOCHROMATOSIS, Joanna L. Hansen, James P. Kushner Univ. of Utah Medical Ctr, Salt Lake City, Utah 84132 Iron homeostasis is perturbed in hereditary hemochromatosis, an autosomal recessive trait characterized by excess iron absorption. The gene responsible for this disease is believed to map to the class I region of the HLA locus on the short arm of chromosome six. This region of the HLA locus has been examined in several pedigrees with hemochromatosis by hybridization analysis of Southern blots. Genomic DNA from seven pedigrees was hybridized with three probes originating in the HLA class I region. Polymorphisms were detected for six different restriction endonucleases with these probes. The inheritance of these polymorphisms were compared with the genotype assigned by HLA haplotyping and clinical analysis of iron stores. The catalogue of restriction fragment length polymorphisms appears to be as useful as HLA typing for identifying homozygotes within a pedigree. Characterization of polymorphisms in the HLA region should facilitate the isolation of the gene responsible for hereditary hemochromatosis.

Z 305 A COMPARISON OF THE BIOSYNTHESIS OF POLY(γ-GLU-CYS)$_n$GLY AND RELATED COMPOUNDS IN CADMIUM-RESISTANT AND CADMIUM-SENSITIVE DATURA INNOXIA CELLS, Paul J. Jackson, Nigel J. Robinson and Emmanuel Delhaize, Los Alamos National Laboratory, Los Alamos, NM 87545.
The effect of Cd on (γ-Glu-Cys)$_n$Gly biosynthesis, protein and RNA synthesis and GSH concentrations was measured in two cell lines of D. innoxia with differing Cd-tolerances. Resistant cells synthesized 40% more (γ-Glu-Cys)$_n$Gly than sensitive cells as early as one hour after exposure to 250 μM Cd. Although sensitive cells died on prolonged exposure to 250 μM Cd, the difference in (γ-Glu-Cys)$_n$Gly production preceded any effects of Cd on rates of RNA and protein synthesis and cell viability in sensitive cultures. The relative overproduction of (γ-Glu-Cys)$_n$Gly in resistant cells was not due to a higher endogeneous supply of GSH nor was resistance associated with exclusion of Cd from cells. The distribution of Cd within cells also differed. Sensitive cells had a proportion of the Cd in low molecular weight forms which were absent from resistant cells. Pulse-labeling experiments with [^{35}S]-cysteine suggested that cysteine supply might be the limiting factor in polypeptide synthesis. These results suggest that the relative overproduction of (γ-Glu-Cys)$_n$Gly by resistant cells is responsible for Cd-tolerance. However, the ability to produce larger amounts of these polypeptides may be related to the amount of precursor(s) available and not to the activity of enzyme(s) directly responsible for these metal-binding polypeptides' biosynthesis.

Metal Ion Homeostasis: *Molecular Biology and Chemistry*

Z 306 A CONVENIENT RAPID ASSAY FOR METALLOTHIONEIN USING CHELEX-100. K. B. Jacobson, K. V. Sloop, L. R. Shugart, L. Adams, B. D. Jimenez and J. F. McCarthy. Biology and Environmental Sciences Divisions, Oak Ridge National Laboratory, Oak Ridge, TN 37831.

Low molecular weight (MW) metal-binding proteins (MBPs) may provide protection against certain toxic divalent metal ions; metallothionein is one member of the MBPs. Since the MBPs are inducible, those in fish liver are potentially useful to indicate whether the fish has been exposed to certain metals recently. A common method for detecting low MW MBPs is by molecular exclusion chromatography. We describe here an alternative method that gives an equivalent measure of the quantity of MBPs in a liver extract. The method consists of a) acid-induced removal of protein-bound metals, b) separation of high MW proteins by isoelectric precipitation, c) tagging the low MW proteins with cadmium-109, and d) subsequent isolation of radiolabeled proteins by chromatography on pasteur-pipette columns containing Chelex-100. This assay was compared to the molecular exclusion chromatography assay and showed excellent agreement for MBPs in livers of Lepomis auritus, the red-breasted sun fish. (Research sponsored by the Office of Health and Environmental Research, United States Dept. of Energy, under contract DE-AC05-84OR21400 with the Martin Marietta Energy Systems, Inc. and funded in part by the ORNL Director's R&D Program.)

Z 307 COPPER TRANSPORT AND PLASMID MEDIATED COPPER RESISTANCE IN *Escherichia coli*, B.T.O. Lee, D. Rouch and J. Camakaris, Department of Genetics, University of Melbourne, Parkville, Victoria, 3052, Australia.

An experimental analysis of copper transport in *Escherichia coli* together with a consideration of copper as both a toxic agent and a biological requirement have been used to construct a model for copper metabolism in *E. coli*. The model consists of 5 major components (1) Copper uptake (2) Cytoplasmic transport, storage and utilization of copper (3) Copper efflux (4) Cellular damage by copper and its limitations, and (5) Regulation of internal copper levels.

The properties of mutants altered in each component of the model can be predicted. The analysis of the mutants already isolated namely, copper sensitive and/or copper dependent indicates five mutant types, which we have named *cut* (copper transport) A-F. These mutants are defective in various copper transport functions including uptake, efflux and storage and have been assigned as follows:

cut A non specific copper uptake; *cut B* copper specific uptake; *cut C* copper efflux; *cut D* copper efflux; *cut E* intracellular copper storage; *cut F* intracellular copper carrier.

Z 308 CADMIUM TOLERANCE IN TRANSGENIC TOBACCO SEEDLINGS EXPRESSING MOUSE METALLOTHIONEIN
Indu B. Maiti, George J. Wagner and Arthur G. Hunt
Plant Physiology/Biochemistry/Molecular Biology Program, Department of Agronomy, University of Kentucky, Lexington, KY 40546-0091

A binary plasmid was constructed to contain the mouse metallothionein c-DNA, the constitutive 35S promoter from cauliflower mosaic virus, the polyadenylation signal from the pea rbcS-E9 gene and several selectable markers. The plasmid was transferred to *Agrobacterium tumefaciens* and the leaf disc method was used to transform tobacco. Callus and shoots were regenerated in the presence of kanamycin and transformed plants were obtained. Southern, Northern and Western blot analysis demonstrated integration and expression of the metallothionein gene in transformed callus and transgenic plants. The gene is transmitted to and expressed in seed derived progeny as a dominant Mendelian trait.

Seeds from self-fertilized transgenic plants germinate and form seedlings in the presence of 1mM $CdCl_2$, whereas those from control, non-transformed plants germinate but do not form seedlings. Transformed seedlings are more tolerant to high Cd than non-transformed controls as expressed by leaf development and number. Responses of immature and mature transgenic plants to Cd challenge are now being tested. Results are consistent with increased tolerance to Cd in transformed seedlings resulting from the expression of metallothionein.

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 309 COORDINATION CHEMISTRY AND SPECIATION OF Al(III) IN BIOLOGICAL SYSTEMS, Arthur E. Martell and Ramunas J. Motekaitis, Department of Chemsitry, Texas A&M University, College Station, Texas, 77843-3255 U.S.A.

There are several conditions, in addition to the dialysis encephalopathy syndrome, that lead to the accumulation of toxic levels of aluminum in the body. However the reactions of aluminum in aqueous biological systems are not well understood. A review of the literature on the coordination chemistry of Al(III) in aqueous solution reveals the existence of a considerable amount of stability constant data of questionable validity. In many cases the strong hydrolytic tendencies of the Al(III) ion have not been considered in data processing and in the interpretation of the complex species present in solution. In this paper equilibrium data are analyzed for the Al(III) complexes of aminopolycarboxylate ligands such as EDTA and its analogs, for the α-hydroxycarboxylic acids, and for catechol and catechol derivatives. Special attention is given to recent results on hydroxy acids such as citric acid, and the involvement of the α-hydroxy group in the coordination of Al(III). The design of ligands suitable for detoxification of Al(III) in physiological systems will be discussed.

Z 310 MECHANISM OF METAL DETOXIFICATION: METALLOTHIONEIN-TYPE STRUCTURES OF THE NEMATODE CUTICLE, Matthew Ryuntyu, Department of Agronomy and Soil Science, University of New England, Armidale. N.S.W. 2351.

Kagi and Nordberd (1979: Proc. 1st Int. Meeting on Metallothioneins, Zurich:34) showed that metal binding proteins (metallothioneins) are involved in detoxification mechanisms by some crystalline structures. TEM cuticle striations are sagittal sections and reveal typical crystalline line arrangements. Because of the close bond between molecules, they result in a very tough protein. Using experimental data of the collagen-like spirals, can be seen as most TEM cuticular structures, morphologically dense and lying in a jelly-like substratum, becoming more "dense" according to the laws of physics for liquid crystal strata. The molecular weight of the collagen components of the nematode cuticle shows that it is composed of at least 18 types of protein ranging from 26,000 to 250,000 Daltons. They are related to metallothionein-type proteins on the basis of copper and cadmium binding. The striation-like type of the cuticle structures is typical of parasitic nematodes living in "difficult" enzymatic environments as it is precisely this structure which makes it resistent to toxic chemicals.

Z 311 THE PRESENCE OF A METALLOTHIONEIN-LIKE PROTEIN IN BOVINE RETINA, T. Takahashi, V. K. Paliwal, and M. Ebadi. Dept. of Pharmacol., Univ. Neb. Coll. of Med., 42nd St. and Dewey Ave., Omaha, NE 68105

Mammalian retinas, which contain photoreceptor systems and initiate electrical impulses on illumination, possess the highest concentrations of zinc in any known living tissues. For example, nocturnal animals with keen vision, such as foxes, have been reported to exhibit a zinc level of 138,000 ppm or 13.8% by weight in the iridescent layer tapetum lucidum of the choroid. Furthermore, zinc deficiency has been implicated in impaired night vision and zinc excess has been detected in inherited retinal dystrophy. The conversion of retinol to retinaldehyde, which in turn is necessary for the formation of rhodopsin (visual pigment), is a zinc-dependent process and is impaired in zinc deficiency state.

In order to study the metabolism of zinc in the retina further, we have measured its subcellular distribution in the bovine retina and found it to be nonuniform, exhibiting the following concentrations (μg zinc/mg protein): rod outer segment (0.230), pre-pellet (0.090), pellet 1 (0.119), pellet 2 (0.091), and post-pellet 2 (0.038). In addition, the bovine retina contains a low molecular weight metallothionein-like protein which exhibits an elution volume (Ve/Vo) of 1.9 on gel permeation chromatography, and produces only one isoform, which on reverse phase HPLC exhibits a retention time of 16.22 min and is similar to that produced by zinc-induced hepatic metallothionein II. The precise compartmentation of the retinal metallothionein-like protein is being investigated at this time. (Supported in part by a grant from USPHS ES-03949.)

Metal Ion Homeostasis: Molecular Biology and Chemistry

Z 312 CHARACTERIZATION OF ZN-BINDING PEPTIDES ASSOCIATED WITH A DECLINE DISORDER OF CITRUS. Kathryn C. Taylor, L.Gene Albrigo and Christine D. Chase, University of Florida, Gainesville FL 32607.
Citrus blight is a decline disorder of unknown etiology. The earliest symptom of the disorder is a redistribution of Zn within the tree. The canopy exhibits symptoms of Zn deficiency while Zn accumulates in the phloem. The phloem Zn is associated with a complexing agent.

A 4 kilodalton, highly anionic, zinc binding fraction was isolated from phloem tissue of blight-affected 'Valencia' sweet orange (Citrus sinensis (L.) Osbeck) and 'Marsh' grapefruit (C. paradisi Macf.) trees. QAE-Zeta chromatography revealed the Zn binding fraction contained at least four different anionic species. Two of the four species were present in tissue from healthy as well as blight-affected trees. The other two species were found in only the blight-affected trees. Amino acid analysis was performed on each of the partially purified Zn-binding factors. The four factors had very similar amino acid compositions and contained a high percentage of asp/asn residues, indicating they are not poly(gamma-glutamyl-cysteinyl)-glycine peptides. Further characterization of these peptides and their metal binding properties is underway.

Z 313 THE ROLE OF METAL-BINDING COMPOUNDS IN THE COPPER-TOLERANCE MECHANISM OF *SILENE CUCUBALUS*, Jos A.C. Verkleij[1], Paul L.M. Koevoets[1], Jan van 't Riet[2], Mary C. van Rossenberg[2] and Wilfried H.O. Ernst[1]. Department of Ecology and Ecotoxicology[1] and Biochemical Laboratory[2], Free University, P.O. Box 7161, 1007 MC Amsterdam, The Netherlands.

Various mechanisms have been proposed to explain the metal tolerance in higher plants. These include: exclusion of metals from the plant, compartmentation of metals in vacuoles and cell walls, evolution of metal-tolerant enzymes and specific metal-binding proteins.

According to Grill *et al.*, 1987 (PNAS 84, 439-443) only metal-binding peptides (phytochelatins) are involved in the detoxification of heavy metals in higher plants. However, the existence of higher Mol. Wt. metal-binding proteins cannot be disregarded although the described high Mol. wt. forms may be assiciation artefacts.

In *Silene cucubalus*, a species occurring on heavy metal contaminated soils, a number of tolerant and non-tolerant strains has evolved. In our study of the molecular basis of Cu-tolerance we characterized the metal binding compounds, synthesized in tolerant and non-tolerant plants after 20 days of Cu-treatment.

We could demonstrate by gelfiltration on FPLC-Superose-12 in root extracts from both types of plants, grown on 40 µM Cu, two compounds: one with an apparent Mol.Wt. of 12 kD and containing more than 60% of the Cu, and another one with a Mol.Wt. of 3.2 kD. After further purification by ion-exchange chromatography and gelfiltration on FPLC, the same Cu-containing compounds appeared.

These results indicate that both tolerant and sensitive plants induce the synthesis of Cu-binding compounds with apparently the same Mol. Wt. upon growth in the presence of high amounts of Cu. Although in both types of plants these compounds may play a role in Cu-detoxification and homeostasis, further research is necessary to elucidate their significance in the Cu-tolerance mechanism. Preliminary aminoacid analysis of this material did not reveal a striking similarity with the known composition of phytochelatins or metallothioneins as yet.

Metal Metabolism and Utilization

Z 400 INDUCTION OF A ZINC BINDING PROTEIN DURING THE EARLY EMBRYONIC DEVELOPMENT OF THE BRINE SHRIMP ARTEMIA SALINA, Roger A. Acey, Dept. of Chemistry, California State University, Long Beach, Long Beach,CA 90840.

We have been able to identify three metal binding activities in Artemia. This was accomplished by pre-incubating cytosol with ^{109}Cd (or ^{65}Zn) and fractionating the mixture on G-75 (or G-50). Metal binding activities were identified by assaying the column fractions for radioactivity. A small molecular weight metal binding activity is clearly evident (MBPI). MBPI is also associated with significant amounts of zinc. Zinc levels were determined by atomic absorption spectrophotometry. The cadmium and zinc binding activities can be resolved by ion-exchange chromatography. A second zinc binding activity (ZnBPII) is present in 24 hr nauplii and increases in concentration during the next 24 hrs. of development. We report here that ZnBPII is actually detectible after 12 hours of development and increases steadily in concentration. Gel filtration on G-50 indicates the molecular weights of MBPI and ZnBPII to be ca. 2000 and 8000 daltons respectively. An interesting aspect of these experiments is that the ^{109}Cd used to detect the position of MBPI undergoes significant changes in distribution between the various protein pools with embryos of increasing age. In cytosols obtained from early stage embryos, the majority of label is associated with the proteins eluting in the void volume of the column. However, with cytosols from older embryos, the label is increasingly distributed between MBPI and ZnBPII with little of the isotope being assoicated with the proteins eluting in the void volume. This work was supported by NIH grant 1 R15 GM36117-01

Z 401 COPPER-RESISTANCE IN CULTURED CHINESE HAMSTER OVARY CELLS, J. Camakaris, J. Patton and D.M. Danks*, Department of Genetics, University of Melbourne, Parkville 3052 Victoria, Australia; *Murdoch Institute, Royal Children's Hospital, Parkville 3052 Victoria, Australia.

Copper-resistant (Cu^R) cultured Chinese Hamster Ovary (CHO) cells have been isolated using various selection protocols. Unlike cadmium resistant CHO cells, the mechanism of resistance does not involve enhanced production of the heavy metal sequestering protein, metallothionein. Cu^R cells accumulate significantly less intracellular Cu than normal CHO cells when incubated in medium containing high Cu concentrations. Overt gene amplifications as evidenced by presence of "Homogeneously Staining Regions" and "Double Minute" chromosomes were not observed although one of the Cu^R variants possessed an extra chromosome whose size correlated with the level of resistance. The Cu^R phenotype was codominant in one of the variants whilst it was recessive in another variant. Efflux kinetics appeared to be normal. Cu uptake was shown to be a saturable process and although the variants had similar apparent Km values the apparent Vmax. was reduced in two of the Cu^R variants. The latter finding may in part explain the Cu^R phenotype.

Z 402 FEPB, A PERIPLASMIC IRON TRANSPORT PROTEIN OF ESCHERICHIA COLI. Charles F. Earhart and Margaret Elkins, The University of Texas at Austin, Austin, Texas 78712.

The product of the *Escherichia coli fepB* gene is a periplasmic protein (FepB) that is required for enterobactin-mediated iron transport. Characterizing FepB is essential for understanding the enterobactin system. Four *fepB*-regulated polypeptides, with apparent molecular weights ranging from 36,500 - 31,500, are detectable in standard SDS-polyacrylamide gels; the three smaller polypeptides are localized in the periplasm. Deletion analyses showed that removal of as little as 17 amino acids from the carboxy terminus of FepB resulted in the appearance of only one polypeptide band. The smallest polypeptide is absent in minicells treated with DNP or CCCP. The *fepB* gene has been sequenced. The unprocessed form of FepB has a calculated Mw of 34,116 and, as anticipated from the above results, a leader sequence (approximately 26 amino acids) is present. The basis for the multiplicity of FepB forms remains unclear but cannot be the result of disulfide bond arrangements as FepB contains no cysteine residues. Also unclear is the significance of a sequence centered approximately 100 bp upstream of the FepB translational start site that is able to form a large stem and loop structure.

Z 403 CYTOFERRIN: CELLULAR LEVELS VARY WITH IRON SUPPLY AND DEMAND. RL Jones and RW Grady, Rockefeller University, New York, NY, 10021.

Cytoferrin (CF) is a low molecular weight iron-binding substance which is elevated in the serum in iron overload or hepatic damage. CF is quantitated by a bacterial growth assay which reflects iron transport. Since CF has not yet been defined structurally, CF activity is expressed relative to that of desferrioxamine (DFO). Despite its presence in serum, the extraction of CF from tissues and cultured cells indicates that its primary location is intracellular. We measured CF levels in several types of cells, some of which were treated so as to modulate their iron metabolism. CF levels were expressed as DFO equivalents/mg of cytosolic protein (ng/mg). Mature red blood cells contained only trace amounts of CF (0.2 ng/mg) while reticulocytes and chick erythrocytes contained 2.6 and 2.8 ng/mg, respectively. Other cells contained significantly greater quantities of CF: J772, 75; MEL, 118, HeLa, 64; Friend, 115; and K562, 139 ng/mg. To investigate the physiologic role of CF, we incubated K562 cells in either 10 uM DFO or 6 uM ferric ammonium citrate, control cultures being left untreated. CF content increased an average of 28% in cells grown in the presence of DFO while decreasing 39% in the presence of the iron salt. Friend cells were grown in the presence of varying concentrations of DMSO, a promoter of differentiation. Both heme and CF content of the cells increased linearly as the concentration of DMSO increased, 2% DMSO causing CF levels to double. Heme and CF were unchanged in a DMSO resistant mutant. Production of CF relative to iron supply/demand suggests an integral role for this compound in cellular iron transport and homeostasis.

Z 404 EFFECT OF HEAVY METALS ON PROTEIN SYNTHESIS IN HUMAN HEPATOMA CELL LINES,

Arturo Leone,Maria Pascale and Paolo Remondelli,University of Naples,Naples,ITALY 80131.

The liver plays a dual fundamental role in the homeostasis of Cu,Zn and Cd providing both the deliver of such essential elements to the other tissues through carrier serum molecules i.e. ceruloplasmin, and their excretion after coniugation with biliary salts.We are studying in our laboratory the effect of these metals on protein synthesis of two highly differentiated hepatoma cell lines, Hep G2 and Hep 3B.Cells were incubated for ten hours with increasing concentrations of CuSO4,ZnCl2 and CdCl2; the analysis of the 35S-Cysteine and 35S-Methionine labelled proteins after a ninety minutes pulse revealed on SDS-Poliacrilammide gel electrophoresis: i) addition of metals to the growth media inhibits protein synthesis; ii) Cd and Zn are the strongest inducers of metallothionein synthesis in Hep G2 and Hep 3B; iii)heath shock like proteins - Hsp70 - appear in response to high doses of metals; iv) new proteins appear after induction with copper,zinc and cadmium.Exposure of both cell lines to heath or inducers of the acute phase response - as the supernatant of lipopolysaccharide stimulated monocytes - does not produce the synthesis of such proteins.One of them has a MW 15,000 daltons , a high content of cysteine and is induced by copper and cadmium only in Hep 3B cells.

Z 405 VARIATIONS IN HEPATIC METALLOTHIONEIN AND ZINC LEVELS AFTER ESTRADIOL TREATMENT OF RAINBOW TROUT

Per-Erik Olsson[1], Muhammad Zafarullah[2], and Lashitew Gedamu[2]
[1]Department of Zoophysiology, Goteborg, Sweden. [2]Department of Bioscience, Calgary, Canada.

The biological role of metallothionein has been subjected to numerous investigations, but the physiological function is still unclear. Metallothionein appears, however, to be involved in the metabolism of zinc and copper. Several investigations have focused on the normal variations in metallothionein content of the liver during fetal and neonatal development in mammals. In a recent study it was demonstrated that metallothionein may be involved in the regulation of zinc during the annual reproductive cycle in rainbow trout. The objective of the present study was to determine the variations in hepatic metallothionein, metallothionein mRNA, vitellogenin mRNA and zinc levels, in rainbow trout, after intraperitoneal injections of estradiol. The induction of vitellogenin synthesis by estradiol injections led to an increased hepatic zinc content which in turn was followed by elevated metallothionein and metallothionein mRNA levels. These results indicate that metallothionein constitutes an important factor in zinc regulation during the period of exogenous vitellogenesis in rainbow trout.

Z 406 A CLONED SYNTHETIC HUMAN METALLOTHIONEIN RESPONSIBLE FOR INCREASED ACCUMULATION OF HEAVY METALS IN ESCHERICHIA COLI,

F. M. Romeyer, F. A. Jacobs, L. Masson, Z. Hanna and R. Brousseau, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2.

The use of metallothionein as a model protein for the specific binding of heavy metals and protein engineering is being explored. For this purpose, a synthetic DNA coding for human hepatic metallothionein (HMT), fraction MT-2, has been cloned in E. coli under the control of the inducible araB promoter through an in-frame fusion to the end-filled Eco RI site of araB' (in the pING 1 plasmid). Upon induction with arabinose, an arab'-HMT fusion protein is produced. The fusion protein represents 8% of the total protein of E. coli. Without arabinose, no fusion protein is expressed, demonstrating that the expression system is tightly repressed. It has been demonstrated when using the radioisotope ^{109}Cd that the renatured araB'-HMT fusion protein after SDS-PAGE can bind the metal in situ. This clearly shows that the HMT moiety of the fused protein keeps its ability to bind cadmium in vitro. Concurrently, a direct correlation is found between the expression in E. coli of the araB'-HMT and the bioaccumulation of Cd++ in vivo. When the cells are grown in presence of 20 µM of Cd++, the clone expressing araB'-HMT accumulates approximately 5 times more Cd++ than the nonproducing cells. Furthermore, in presence of 1mM Cu+, an increase of bioaccumulation of Cu+ (two fold) has been established when the fusion protein is produced.

Z 407 HEAVY METAL RESISTANCE AND DISTRIBUTION OF VARIOUS METAL IONS IN DIFFERENT STRAINS OF YEAST. V. Santhanagopalan and J. Jayaraman, Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021. India.

A number of yeast (S. cerevisiae) strains were tested for their resistance for different metals. The typical protocol of growing the cells in the presence of various concentrations of Cobalt, Cadmium, Zinc and Copper salts was adopted. The cadmium resistant strains show an accumulation of about 15% of the total cadmium added in the form of cadmium chloride. The results indicate that the Cd-sensitive and Cd-resistant strains differ from each other in the distribution pattern of cadmium in their cells. To look for (a) the cause of resistance, the yeast strains were tested for the uptake of cadmium and for (b) induction of any protein, proteins were analysed on polyacrylamide gel electrophoresis under non-denaturing conditions. Almost all the cadmium present in the soluble fraction was found binding to proteins of high molecular weight. From this, we can say that yeast cells contain a system, which is distinct from Metallothionein to handle the "Cadmium stress".

Z 408 ACCUMULATION OF METALLOTHIONEIN mRNA IN ZINC DEFICIENT RATS M.L. Scarino, M.C. Mellone *, A.Leone*, G.Morelli, Istituto Nazionale della Nutrizione, Rome , *University of Naples, Naples ITALY

In order to follow the induction of the metallothioneins, zinc deficient and control animals were injected intraperitoneally with zinc.Total RNA from liver and kidney was prepared at different times after induction by zinc (0,1,3,6,10,24 hours) and probed with a labelled MT-cDNA .Using a slot blot assay we were able to compare the kinetics of induction between the two groups of animals.In control rats the level of MT-mRNA reaches the maximum after 6 hours and rapidly goes down to the basal level in the next 4 hours. In zinc deficient rats a different pattern of accumulation is observed : the level of MT-mRNA reaches the maximum after 10 hours and remain at high level until 24 hours after the zinc administration Moreover comparing the maximum levels of MT-mRNA in liver and kidney we find a 2.4:1 ratio in zinc deficient and 0.6:1 ratio in the control animals. The amount of zinc/g of tissue increases with time in the liver of both zinc deficient and control rats. Zinc does not accumulate in kidney in both groups.

Z 409 A NEW METHOD FOR QUANTITATIVE ULTRA-TRACE ELEMENT ANALYSIS OF MICROGRAM SAMPLES, Harold W. Schmitt, N. Thonnard, Larry J. Moore, James E. Parks and Robert D. Willis, Atom Sciences, Inc., Oak Ridge, TN 37830, and K. Bruce Jacobson, Oak Ridge National Laboratory, Oak Ridge, TN 37830.

New levels of sensitivity in elemental and isotopic analysis have become possible in recent years by combining resonance ionization with mass spectrometry. The technique, Resonance Ionization Spectroscopy (RIS), utilizes one or more tunable, narrow-band lasers which can be tuned to specific transitions of the selected element. Atoms of that element are sequentially excited to specific excited states chosen for their uniqueness to the selected element, then finally ionized. The ions can then be passed through a mass spectrometer for isotopic determinations and counted with conventional charged particle detectors. The process is extremely selective, virtually interference-free, because of the uniqueness of the selected excited states and transition energies. With proper lasers the method is extremely sensitive, since individual atoms of the selected element can be identified and counted. Finally, the technique is general, as all elements in the periodic table (except He and Ne) can be analyzed. This method allows significant simplification in sample preparation. A beam of ions can be used to atomize a sample surface (sputtering); by focusing the sputtering ion beam to a suitable size, e.g. a few micrometers, and rastering it over the sample, the trace element distribution can be imaged, while depth profiling is possible by collecting data as the sample is eroded. Data of various types will be shown for biological, environmental and solid-state samples. Sensitivities achieved to date are at the ppb level in solids, picograms in liquids, and 0.1 atoms per cc for noble gases.

Z 410 HIGH RESOLUTION ACIDIFICATION KINETICS OF TRANSFERRIN IN BALB/c 3T3 AND K562 CELLS: DIFFERENCES IN ACIDIFICATION AND ALKALINIZATION, David M. Sipe, Alexander Jesurum, and Robert F. Murphy, Department of Biological Sciences & Center for Fluorescence Research in Biomedical Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213. The kinetics of transferrin acidification were determined for both Balb/c 3T3 and K562 cells using a dual laser flow cytometer and transferrin labeled with fluorescein (F) and lissamine rhodamine (LR) dyes. Cells were labeled at 4°C, then warmed to 37°C to initiate synchronous endocytosis. Average values for both F and LR signals were calculated for single-cell data acquired over 25-60 min. The fluorescence of transferrin remaining on the cell surface was subtracted from the total green and red fluorescence signals, allowing calculation of a ratio for only the *internalized* transferrin. Comparison of this ratio with calibration data allowed calculation of average internal pH. Both cell lines began endocytosis and rapid acidification of transferrin within 3 min of initial warm up. However, the cell lines differed both in the extent of acidification and in subsequent treatment of the transferrin after achieving a minimum pH value. For Balb/c 3T3 cells, the lowest pH observed was above 6.0, occurring 5 min after warmup. The rapid initial acidification was followed by a slower alkalinization to external pH, occuring with a half time of 5 min. In contrast, for K562 cells the minimum pH was below 5.5. No alkalinization was observed, even though transferrin appeared to recycle and dissociate normally. The results suggest that the pH to which transferrin is exposed by these cell types may be related to their iron requirements *in vivo.* We are investigating the basis for this difference in acidification, and possible differences in iron delivery to these cell types. We are also determining the ionic requirements and temperature dependence of transferrin acidification. (Supported by NIH grant GM32508 and training grant GM08067, and NSF DCB8351364.)

Z 411 CADMIUM TOLERANCE IN TRANSGENIC TOBACCO SEEDLINGS EXPRESSING MOUSE METALLOTHIONEIN

Indu B. Maiti, George J. Wagner and Arthur G. Hunt
Plant Physiology/Biochemistry/Molecular Biology Program, Department of Agronomy, University of Kentucky, Lexington, KY 40546-0091

A binary plasmid was constructed to contain the mouse metallothionein c-DNA, the constitutive 35S promoter from cauliflower mosaic virus, the polyadenylation signal from the pea rbcS-E9 gene and several selectable markers. The plasmid was transferred to *Agrobacterium tumefaciens* and the leaf disc method was used to transform tobacco. Callus and shoots were regenerated in the presence of kanamycin and transformed plants were obtained. Southern, Northern and Western blot analysis demonstrated integration and expression of the metallothionein gene in transformed callus and transgenic plants. The gene is transmitted to and expressed in seed derived progeny as a dominant Mendallion trait.

Seeds from self-fertilized transgenic plants germinate and form seedlings in the presence of 1mM $CdCl_2$, whereas those from control, non-transformed plants germinate but do not form seedlings. Transformed seedlings are more tolerant to high Cd than non-transformed controls as expressed by leaf development and number. Responses of immature and mature transgenic plants to Cd challenge are now being tested. Results are consistent with increased tolerance to Cd in transformed seedlings resulting from the expression of metallothionein.