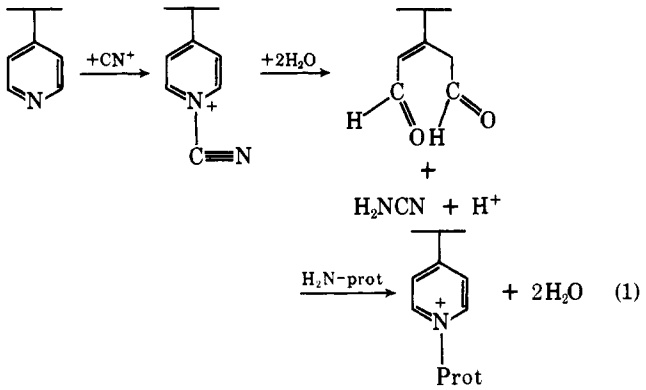
Polymers containing pyridine can be directly converted into polyaldehydes (glutaconic aldehyde) by the reaction with cyanogen bromide. The polyaldehyde formed can be used for the binding of protein and other ligands. The overall reaction is summarized in the eq 1. In these polymers no cross-linking can



(1)

occur, and the amount of aldehyde formed is controlled by the amount of cyanogen bromide added. Further advantages are that excess aldehyde groups can either be reduced with borohydrides or converted back to pyridine by reaction with ammonia. Also the products of the reaction with proteins are pyridinum salts and therefore they are completely stable and no leakage was observed on prolonged storage.

In order to show that polypyridines can be converted into the corresponding aldehydes, polyvinylpyridine, polysaccharides, glass beads, and polyacrylamide derivatives containing pyridine were prepared, and reacted with CNBr under anhydrous conditions. The polymers were filtered and treated with water followed by reagents which react with aldehyde. All the pyridine-containing polymers gave strong blue colors with barbituric acid and reacted strongly with *p*-nitrophenylhydrazine, indicating the presence of aldehydes. This was substantiated by infrared spectroscopy.

The aldehydes formed were also capable of binding amines. This was demonstrated by the binding of the copper complex of lysine through its ϵ-amino groups. The amount of lysine bound to polyvinylpyridine was 1.3 mmol/g of dry gel as determined by the amount of copper bound. All of the polymers described above were used to bind several proteins including trypsin and chymotrypsin and specific examples of enzyme binding are given below.

(1) Dry polythiol (Koch & Light), 1.0 g, was heated with 4-vinylpyridine in a well-stoppered flask on a shaker for 3 h at 37 °C. The suspension was then kept at room temperature overnight. The gel was filtered, washed carefully with ethanol, and dried. The gel was reacted at room temperature with 1–3 g of CNBr in dry dioxane (5 mL) for 5 min. Water was then added and the mixture stirred for 20 min at room temperature. The gel was filtered, washed with cold water, and stirred overnight with a solution of trypsin in phosphate buffer pH 5.0 at 4 °C. Using this procedure one can bind up to 117 mg of enzyme/g of dry gel. The specific activity of the immobilized enzyme was 70% that of soluble trypsin. The apparent K_M in the hydrolysis of (N,α)-benzoylarginine ethyl ester was close to that of soluble trypsin. The pH optimum for activity of the immobilized enzyme was about pH 9.5.

(2) Glass beads containing amino groups, 1.0 g,[10] were reacted with pyridine-4-carboxyaldehyde in phosphate buffer (pH 7.0 for 1 h at room temperature), washed with water, and dried carefully. The beads were suspended in dioxane (5 mL), degassed, and activated with 1–3 g of CNBr dissolved in absolute dioxane (3 mL) for 5 min at room temperature. Following the addition of 0.25 M carbonate buffer pH 9.0, the mixture was stirred at room temperature for 20 min, the pH being held constant by manual titration with 2 N NaOH. The activated glass beads were then washed with cold water and coupled to the enzyme in the same manner as described above. Trypsin, as well as α-chymotrypsin bound to glass matrices, had high specific activities. The pH optimum for activity of the immobilized trypsin was the same as with the other gel, whereas that of α-chymotrypsin was pH 9.0.

The polypyridines described are not the only polymers that can be used since the number of methods for preparing pyridine containing polymers and copolymers is almost unlimited judging from the numerous pyridine derivatives available. Pyridine is also a component of many synthetic membranes and ion-exchange resins. Thus, proteins as well as other molecules, can be bound to any of these polymers.

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Highly Selective Membrane Transport of Pb^{2+} from Aqueous Metal Ion Mixtures Using Macrocyclic Carriers[1]

Sir:

Incorporation of macrocyclic ligands into hydrophobic membranes to serve as cation carriers offers a method to exploit the high cation selectivity[2] demonstrated by these ligand molecules. Means have been developed[3,4] whereby cation transport of this type can be coupled to free-energy gradients which drive the flux of cations against the cation concentration gradient. Potential applications[5] of this technology include the separation and concentration of chemical species, the detection and measurement of chemical species, and the removal of undesirable chemical species from the environment or from biological systems.

In the present communication, we report the transport rates of Pb^{2+} and of several alkali, alkaline earth, and transition metal cations through liquid membranes containing one of several macrocyclic ligands. Our objective was to determine the effectiveness of membranes containing macrocycles in selectively transporting Pb^{2+} which is of interest in relation to the environment and human toxicity. Some membrane transport data have been published indicating rates of transport of individual cations.[3,4,6-10] In addition, Tl(I) and K^+ have been transported by macrocycles in the presence of a large excess of Na^+.[11] However, we believe that this is the first report of liquid membrane transport in which macrocyclic carriers are used to effect separation of divalent metal cations from cation mixtures. We have found that remarkably high transport selectivities for Pb^{2+} can be achieved even when the ratio of Pb^{2+} concentration to the concentration of another cation in the mixture is $<1/100$.

Liquid membrane experiments were performed using cells

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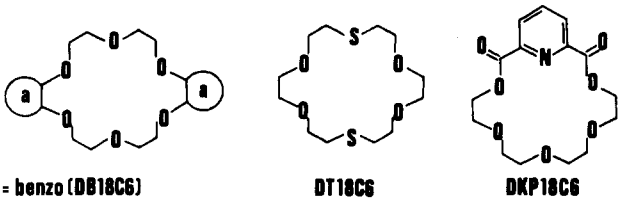
Table I. Moles of Pb^{2+} and M^{n+} Transported Simultaneously from an Aqueous Solution through a Chloroform Membrane Containing One of Four Macrocyclic Carriers or BAL

source phase,[a] Pb^{2+}/M^{n+}	ratio of moles transported[b] × 10^7				
	DC18C6	DT18C6	DB18C6	DKP18C6	BAL
Pb^{2+}/Li^+	300/0	160/0	14/0	280/0	
Pb^{2+}/Na^+	240/1.0	77/0.5	7.0/1.6	270/1.2	
Pb^{2+}/K^+	270/2.4	73/0.5	7.0/53	210/9.3	
Pb^{2+}/Mg^{2+}	360/0.2	120/0.2	18/0.2	220/0.1	0.01/0.1
Pb^{2+}/Ca^{2+}		180/0.2	15/0.2	370/0.5	0.03/...
$Pb^{2+}Sr^{2+}$	330/6.4	120/0.01	11/0.07	270/1.3	0.02/0.004
$(Pb^{2+}/Ba^{2+})^a$	260/2.9	27/0.02	1.1/0.04	100/0.02	0.02/...
$Pb^{2+}Fe^{3+}$	340/0.07	240/0.07	27/0.10	400/0.06	0.10/0.25
Pb^{2+}/Cu^{2+}	250/0.02	160/0.02	13/0.01	330/0.01	0.03/0.01
Pb^{2+}/Zn^{2+}	320/0.06	140/0.05	21/0.06	250/0.12	

[a] Source phase is 0.5 M $Pb(NO_3)_2$ plus 0.5 M $M(NO_3)_n$ in water, except where $M^{n+} = Ba^{2+}$, for which 0.15 M of each salt was present. [b] Numbers are moles transported per 24 h reported as the average of three independent determinations; reproducibility, ±20% or better.

which were smaller versions of those described previously.[10] The cells consisted of a 3-mL membrane phase (reagent grade chloroform containing 0.001 M carrier, stirred at 120 rpm by magnetic stirrer), interfaced to both a 0.8-mL source phase (salt solution) and a 5.0-mL receiving phase (distilled deionized water). After a period of 24 h, a 4-mL sample of the receiving phase was withdrawn and the number of moles of each cation was determined by atomic absorption spectroscopy. Several ligands were used as cation carriers. Of these, results for a few representative ligands are reported here: dicyclohexano-18-crown-6 (mixture of isomers) (DC18C6), 1,10-dithia-18-crown-6 (DT18C6), dibenzo-18-crown-6 (DB18C6), 2,6-



diketopyridino-18-crown-6 (DKP18C6), and 2,3-dimercaptopropanol (BAL). An attempt was made to use the disodium salt of ethylenediaminetetraacetic acid and penicilamine (free base) as carriers, but they were not sufficiently soluble in chloroform. Blank experiments (no carrier present) were performed for each source phase salt solution to determine membrane leakage. The amount of cation leakage varied with cation but was always $<0.2 \times 10^{-7}$ mol/24 h.

In Table I are listed the transport rates of Pb^{2+} and of the cation, M^{n+} which was present with Pb^{2+} in equimolar concentrations in the source phase. Results obtained for Rb^+ and Cs^+ and for several additional carrier ligands are omitted for the sake of brevity. A striking feature of the data in Table I is the almost universal transport selectivity demonstrated by the crown ligands (all of which were based on the 18-crown-6 structure) for Pb^{2+} over the other cations. This selectivity corresponds to the known thermodynamic selectivity of DC18C6 and DT18C6 for Pb^{2+}, as reflected in log K values measured in water.[2] The large transport selectivity for Pb^{2+}, which allowed only minimal flux of any other cation, was unexpected in light of previous transport experiments involving single cation source phases, wherein Na^+, K^+, Sr^{2+}, and Ba^{2+} were transported at significant rates by all five macrocyclic ligands. For example, in single cation source phase experiments, Pb^{2+} was transported at 290×10^{-7} mol/24 h and K^+ at 340×10^{-7} mol/24 h by DC18C6. However, when both are present in the source phase, the mole ratio of Pb^{2+}/K^+ transported is 270/2.4 (Table I). This result is likely due to the effect of the equilibrium constants for the reactions of Pb^{2+} and K^+ with DC18C6, a more complete discussion of which will be presented in a subsequent publication.

The degree of selectivity for Pb^{2+} varies both with carrier and with competing cation. Among the macrocyclic ligands studied, DB18C6 is the least selective for Pb^{2+} and also the least effective carrier in terms of moles of cation transported. It is interesting that this is the macrocyclic ligand most extensively studied as a membrane carrier.[7,8,10] Of the other macrocycles listed in Table I, DC18C6 and DKP18C6 are the most selective for Pb^{2+} and are also very effective Pb^{2+} carriers. These ligands demonstrate effective transport selectivity for Pb^{2+} over the biologically important cations Na^+, K^+, Ca^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+}. Among the cations chemically most similar to Pb^{2+}, i.e., Sr^{2+} and Ba^{2+}, good selectivity for Pb^{2+} is also maintained.

The ligand BAL, used in treatment of lead poisoning, is a very poor transport carrier of Pb^{2+} when compared with any of the macrocyclic ligands in Table I. This may be a result of loss of this water-soluble ligand to the water phases where it decomposes;[12] alternatively the ligand may bind the cations too strongly to release them into the receiving water phase and may simply retain the cations in the chloroform membrane. The latter alternative is substantiated by high log K values[13] for the reaction between BAL and several transition metal cations.

The high degree of transport selectivity demonstrated by the macrocyclic ligands led us to perform transport experiments in which the source phase contained disproportionately large levels of either Ca^{2+} or Sr^{2+} compared with Pb^{2+}. The details of these experiments will be reported at a later date. However, the following results are of interest: (i) The ligand DKP18C6 maintained the most effective $Pb^{2+} > Ca^{2+}$ selectivity over all concentration ranges; i.e., from a source phase containing 1 M $Ca(NO_3)_2$ and 0.01 M $Pb(NO_3)_2$, the mole ratio of Pb^{2+} to Ca^{2+} transported was 41/0.8. (ii) The ligand DC18C6 was most effective for selective transport of Pb^{2+} over Sr^{2+}; i.e., from a source phase 1 M $Sr(NO_3)_2$ and 0.01 M $Pb(NO_3)_2$, the ratio of Pb^{2+} to Sr^{2+} transported was 34/0.6. The various ligand, cation, and system parameters which influence cation transport and which are responsible for this remarkable Pb^{2+} selectivity are currently being studied in our laboratory.

The high degree of transport selectivity for Pb^{2+} demonstrated by the macrocyclic ligands studied, even in the face of large competing concentrations of other cations, has significant implications. Incorporation of macrocyclic ligands which are selective for Pb^{2+} into liquid membranes such as those developed by Li and co-workers[14] may be used to remove Pb^{2+} from environmental and biological systems. In a broader sense, these experiments demonstrate the potential application to selective removal, concentration, or purification of Pb^{2+} or other metallic elements from mixtures.

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^{113}Cd NMR Spectrum of Substituted Horse Liver Alcohol Dehydrogenase

Sir:

In recent months there have been several reports of the use of ^{113}Cd NMR in metalloproteins. It has been reported for carbonic anhydrase,[1] alkaline phosphotase,[2] carboxypeptidase,[3] and concanavalin A.[4] These reports have shown the large chemical-shift range which is possible for ^{113}Cd even when dealing with simple coordinate bonding in biological systems. In this report we extend even further the range of expected ^{113}Cd chemical shifts to +751 ppm with respect to aqueous Cd^{2+}. This has been observed for Cd(II) bound to four cysteine residues in horse liver alcohol dehydrogenase (LADH). In addition, we have observed a second resonance assigned to the catalytic site of this enzyme. Further studies of this site with bound substrates should prove to be a powerful tool in mechanistic studies of this important enzyme.

LADH is a dimeric protein of 80 000 mol wt.[5] This enzyme, together with its coenzyme (NAD^+), catalyzes the oxidation of a wide variety of alcohols. Each of the two identical subunits normally contains two different Zn(II)'s. The "catalytic" Zn(II) is at the bottom of the active site pocket where both coenzyme and substrate are bound.[6] This Zn(II) is coordinated by two cysteine residues and one histidine. The fourth coordination position is presumably occupied, in the absence of a substrate, by an H_2O or an OH^-. The second Zn(II) in each subunit is a "noncatalytic" or "structural" Zn(II) according to kinetic and chemical evidence,[7] and it is coordinated by four cysteine residues in an approximately tetrahedral array. In 1970 Vallee[8] completely replaced the native Zn(II) by either Co(II) or Cd(II) using competitive equilibrium dialysis at pH 5.5. The metal-substituted enzymes retain catalytic activity, although it is reduced over that for the native enzyme. More recently, two methods have been developed[9,10] to replace selectively both kinds of Zn(II)'s. Our sample of LADH had both sites replaced by ^{113}Cd using a modification of Vallee's most recent method.[9]

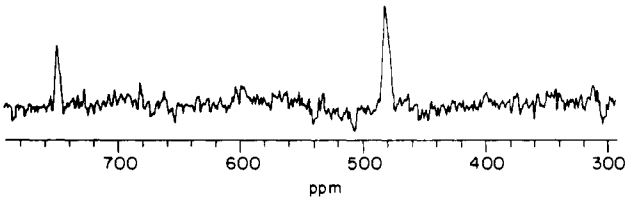

Figure 1. ^{113}Cd NMR spectrum of 90% ^{113}Cd-substituted LADH at 4 °C. The dimeric enzyme concentration was 0.29 mM. The measurement was made on 19 mL of sample with a 25-mm tube in a 180-MHz (^{1}H) magnet. The buffer was 50 mM sodium phosphate, pH* 7.5, with D_2O added to provide an internal lock. The NMR parameters were as follows: acquisition time, 0.2 s; pulse delay, 3 s; pulse angle, 70°; and spectral width, 20 kHz. This spectrum required 24 000 transients. A 45-Hz line broadening was used for sensitivity enhancement. The chemical-shift scale is referenced to 50 mM $CdSO_4$ at 22 °C as 0 ppm.

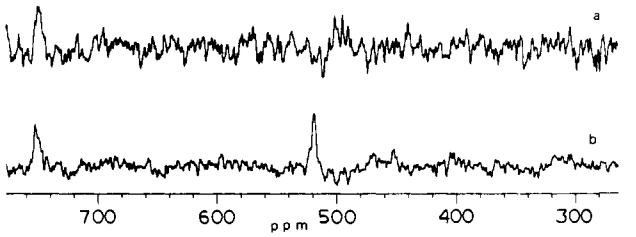

Figure 2. ^{113}Cd NMR spectra of 0.24 mM (dimeric) ^{113}Cd-substituted LADH at 4 °C (the buffer in both cases includes 50 mM sodium phosphate and 10% D_2O): (a) total imidazole concentration 2.4 mM, pH* 7.5; (b) total imidazole concentration 24 mM, pH* 7.8. Part a includes fewer data accumulations than b.

Figure 1 shows the ^{113}Cd NMR spectrum of Cd^{2+}-substituted LADH at pH 7.5. The resonance at 483 ppm is assigned to the catalytic site and the one at 751 ppm to the noncatalytic site. There are two bases for this assignment. The NMR studies of model Cd(II) systems[11,12] show that oxygen-containing ligands are the least deshielding and so aqueous Cd^{2+} is usually used as the 0 ppm reference at the high-field, or really low-frequency, end of the spectrum. Sulfur appears to be the most deshielding of any ligand. Haberkorn et al.[11] observed a deshielding trend for alkylthiolate complexes derived from glutathione and β-hydroxyethanethiol. Deshielding increased sharply with complete sulfur ligation and for $Cd(SR)_2$ it was 318 ppm, for $Cd(SR)_3$ it was 380 ppm, and for $Cd(SR)_4$ it was 520 ppm. Halides and ligands binding through nitrogen have intermediate deshielding effects. Very recently, in metallothionein,[13] chemical shifts in the range of 610–680 ppm have been observed for a coordination which must involve bridging cysteine sulfur. Our assignment appears to be quite certain on the basis of the known coordination of the two sites and the previously observed deshieldings.

An imidazole binding experiment confirms this assignment. It is known that imidazole competes with H_2O for the fourth coordination position of the catalytic Zn(II) in the native enzyme. X-ray crystallography shows imidazole displacing zinc-bound water.[14] In solution it removes the pH dependence of the binding of NAD^+ by apparently blocking the fourth position to H_2O and OH^-.[15,16] Figure 2a shows the ^{113}Cd NMR spectrum with the imidazole concentration five times the catalytic site concentration. The binding constant of the native enzyme for imidazole is fairly small at pH 7.5.[15] The 483-ppm peak cannot be observed, and it is presumably broadened by chemical exchange. The large chemical shifts characteristic of ^{113}Cd can lead to considerable NMR broadening if a dynamic equilibrium is involved. Figure 2b shows the NMR spectrum resulting from addition of a large

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