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Column Chromatography and Kinetics of Nucleosides and Nucleic Acid Bases on Immobilized Nickel– and Cobalt–CDAE–Sporopollenin

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ABSTRACT

Sporopollenin, a natural polymer, has been modified for application as a ligand-exchange material. The ligand-exchange chromatography with the functionalized *Lycopodium clavatum* is a useful method for the rapid separation of nucleosides and nucleic acid bases. The synthesis of tris(carboxymethyl) ethylene diamine *Lycopodium clavatum* has been described. The resin contained functional diaminoethane and carboxyl groups. Nickel(II) and cobalt(II) metal ions can easily be immobilized on this carboxylated-diamino-ethyl (CDAE)–sporopollenin. Physicochemical and chelating properties of *Lycopodium clavatum* have been studied extensively. Ligand-exchange chromatography of CDAE–sporopollenin has also been compared to the conventional synthetic chelex-100 resin. The kinetics of cytidine in CDAE–sporopollenin resin has been investigated. The rate measurements have been made by a potentiometric technique. The relative rates at which nucleosides bind to the resin are determined by the actual chemical-exchange reaction between ligands and resin.

INTRODUCTION

Ion-exchange resins of various types have been used in the past to separate nucleosides and nucleic acid bases (1, 2). About 30 years ago "ligand-exchange chromatography" was introduced as a separation method based on selective adsorption and replacement of amines and other small molecular size solutes from metal ions immobilized on resins

(3). Ligand-exchange reactions have also been studied extensively by Walton (4) and Davankov (5) and their coworkers who compared several resin types and coordinating metals in studies of ligand exchange between amines and ammonia.

The principle upon which ligand-exchange chromatography (LEC) is based is that a transition metal is fixed on a solid support, which can be used for the exchange of bound ligands of the metal. An exchange of ligands takes place between the external solution and the coordination shells of the metal ions in the resin. Ligand exchangers are sensitive to the molecular structure of ligands. Small differences in molecular shape or size often have a large effect on the strengths of binding to a metal-loaded ion exchanger (4). Ligands such as nucleosides and nucleic acid bases can be removed from the solid matrix by the formation of complexes of ammonia with the metal attached to the resin (6).

New developments in ligand-exchange chromatography began with the use of resins with a stronger affinity for metals such as chelex-100, which contains iminodiacetate functional groups (7). Chelex-100 proved to be even better with regard to its ability to keep metal ions, but this type of resin shows slow kinetics (8). Furthermore, another problem with polymer-type resins is their poor mechanical strength. High pressure cannot be used, otherwise the resins deform or collapse, and column permeability decreases. To improve stability, the solid material used must be pressure-resistant.

Cation-exchange resins with carboxylic and phosphonic groups hold their metal ions more tightly and can be used advantageously as stationary phases in LEC (9).

Sporopollenin obtained from *Lycopodium clavatum* occurs naturally as a component of plant spore walls. Sporopollenin exhibits very good stability after even prolonged exposure to mineral acids and caustics. It has important advantages over synthetic resins including the following: constancy of chemical structure, high capacity, chemical stability, uniformity of particle size, and commercial availability (10).

Exchange reactions which deal with the kinetics of the ligand-exchange reactions involving macromolecular resin have been studied. In this paper we investigate the kinetics of the ligand-exchange reaction between water in the resin and cytidine by using cobalt(II)-loaded CDAE–sporopollenin.

EXPERIMENTAL

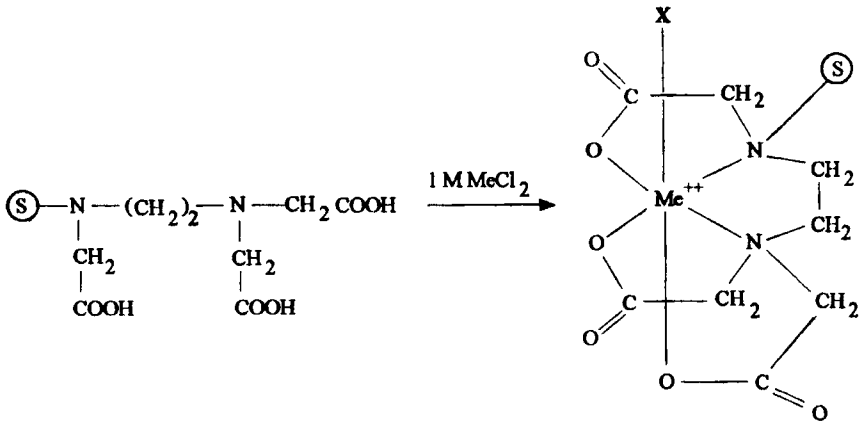
Chemicals and Reagents

Lycopodium clavatum was purchased from Fluka Chemica-Biochemica under the label Lycopodium. Other reagents were purchased from Sigma

Chemical Corp. Solutions were prepared using double-distilled water without further purification by dissolving a weighed quantity of Analar grade material.

Synthesis of Chelating Resins

Lycopodium clavatum was aminated by treatment with 1,2-diaminoethane (DAE) (11). Dried DAE–sporopollenin was placed into beakers. Bromoacetic acid that had been neutralized with 2 M NaOH and 1 M $NaHCO_3$ was added to this resin. After the suspension was stirred for 15 hours at room temperature, the resin was washed with water, diluted with acetic acid, and again washed with water (12). Thereafter, the CDAE–sporopollenin resin obtained was treated with 1 M $NiCl_2$ and 1 M $CoCl_2$ in separate beakers overnight to convert it into the nickel and cobalt forms. Excess $NiCl_2$ and $CoCl_2$ were removed by repeated thorough washing with double-distilled water, and then the resins were kept in 1 M ammonia overnight. The reaction is



Me^{++} loaded carboxylated diamino-ethyl sporopollenin (CDAE-sporopollenin)

Chromatography

All experiments were carried out at room temperature. Columns (30 × 1 cm) were packed with CDAE–sporopollenin resin and chelex-100 resin. In the chromatographic set up, we used a peristaltic pump P1 (Pharmacia Fine Chemicals), and the chromatographic separations were followed by a continuously recording UV spectrophotometer (Shimadzu UV 160 A).

Resin was packed in separate columns in double-distilled water. Each column was then washed with at least one total bed volume of eluant that we use in the adsorption–desorption process in order to ensure that no leakage of the metal would occur during the actual experimental run later on. Samples were injected on the column with micropipets. The elution agent ammonia was pumped, then the effluent was analyzed.

Kinetics

A known quantity of dry CDAE–sporopollenin was placed in contact with a known volume of double-distilled water. The ligand exchanger was stirred until a uniform slurry was obtained. Then a known volume of 0.02 M cytidine was added to the suspension. As a result of ligand exchange with the resin, the solution composition change with time, and this change was monitored by a pH meter (Orion, model SA-720).

RESULTS AND DISCUSSION

It is apparent that ligand-exchange chromatography is an extremely useful technique for the separation of nucleic acid components. Ethylene diamine complexes have very stable structures and cannot be readily dissociated. Stable stationary complexes are produced when resins with carboxylated groups interact with transition metal ions. A difficulty with the ligand-exchange technique is that metal ions are displaced from the column by ordinary ion exchange with the ammonium ions present in aqueous ammonia. This fact has restricted with the choice of metal ions. Ligand exchange with CDAE–sporopollenin has not had this type of problem. Metal cations are strongly bound to the resin and no leakage has been observed. If CDAE–sporopollenin is compared with a cellulosic exchanger, it is obvious that leakage from the cellulosic exchanger is serious because of the small amounts of metal held by these exchangers, and the column soon became white at the top (4). Ni^{2+} and Co^{2+} cations form metal–ligand complexes with the CDAE–sporopollenin. Metal(II)-loaded resin columns are stable over several years. No leaching of the immobilized metal cation was observed. Column effluent was tested several times with dimethylglyoxime which is a sensitive compound for Ni^{2+} determination, and it was seen that eluant was free from nickel(II) ions.

Mixtures of nucleosides and nucleic acid bases are separated with Ni^{2+}-loaded CDAE–sporopollenin as indicated in Table 1(A), and chromatogram 1 is given in Fig. 1. These separations were carried out by linear elution using a carrier of 1 M NH_4OH. A single eluant was employed, and no regeneration of the column is required following the analysis; con-

TABLE 1
Retention Volumes of Nucleosides and Nucleic Acid Bases by Using (A) Ni^{2+}-Loaded CDAE–*Sporopollenin*, (B) Co^{2+}-Loaded CDAE–*Sporopollenin*, (C) Ni^{2+}-Loaded Chelex-100, and (D) Co^{2+}-Loaded Chelex-100 Resin; Elution, 1 M Ammonia; Sample Concentration, 0.02 M

	Chromatogram	Molecule	Wavelength (λ)	Amount injected (μL)	Retention time (min)	Retention volume (mL)
A	1	Guanosine	260	50	16.67	15.6
		Adenosine		50	29.17	27.3
	2	Uracil	254	20	6.83	9.7
		Cytosine		50	9.92	14.0
	3	Uracil	254	40	12.50	6.8
		Cytosine		40	31.67	17.2
		Adenine		40	68.3	37.0
B	4	Uridine	260	30	16.17	13.2
		Adenosine		30	27.17	22.2
	5	Uracil	260	30	16.17	16.1
		Cytosine		30	22.00	22.0
C	6	Uridine	260	30	16.17	13.2
		Adenosine		30	27.17	22.2
D	7	Uracil	260	30	16.17	16.1
		Cytosine		30	22.00	22.0

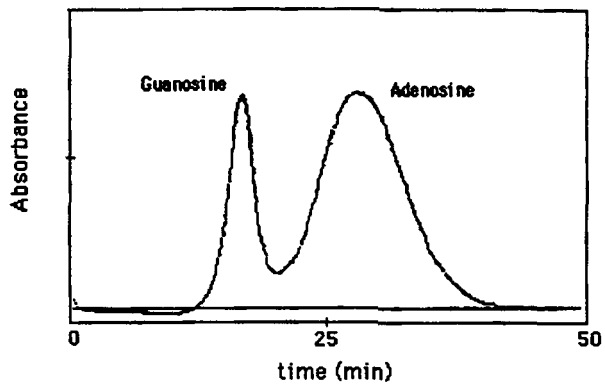


FIG. 1 Separation of nucleosides on the nickel(II)-loaded CDAE–sporopollenin resin: column, 30 × 1 cm; eluent, 1.0 M NH_4OH; flow rate, 0.94 mL/min; detection at 260 nm.

sequently the column is in readiness for another separation. Weakly basic nucleosides such as guanosine are only weakly retained by the column, but they can be separated by elution with diluted ammonia. Nevertheless, the more basic nucleosides are retained more strongly on the column and can be displaced by 1 M ammonia which is a convenient eluting agent. As indicated in Table 1, chromatogram 3, the order of elution from the column is uracil, cytosine, and adenine. Since uracil does not have an amino group, it is not strongly absorbed on the resin. The absence of an imidiasole ring on uracil and cytosine would account for their lesser degree of binding. Molecules which are injected into the column are eluted in the order of decreasing bond strength with the resin.

The result obtained upon passage of a complex mixture of nucleosides and nucleic acid bases through the Co^{2+}-loaded CDAE–sporopollenin resin are shown in Table 1(B), and chromatogram 4 is illustrated in Fig. 2.

Mixtures of nucleosides and nucleic acid bases were separated with nickel- and cobalt-loaded chelex-100 resin as indicated in Table 1(C) and (D), respectively. They can be eluted with a convenient eluting agent, 1 M ammonia. Goldstein and coworkers used LEC for the separation and analysis of nucleic acid components. They used 1 M ammonia solution to desorb nucleosides of higher basicity (13).

The results obtained by using natural and synthetic ligand exchanger were compared. Chelex-100 ligand exchanger resin has some distinct disadvantages. Halide salts and acidic solutions leach metal ions from the resin. Longer columns and slower flow rates give better resolutions (7) whereas CDAE–sporopollenin gives better resolutions even if the column size is small and there are high flow rates, which is convenient, and it

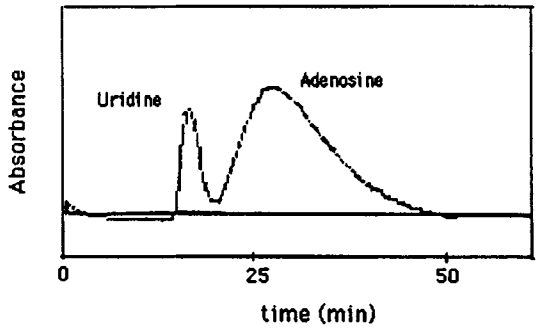


FIG. 2 Separation of nucleosides on the cobalt(II)-loaded CDAE–sporopollenin resin: column, 30 × 1 cm; eluent, 1.0 M NH_4OH; flow rate, 0.82 mL/min; detection at 260 nm.

is very active in the ligand-exchange process. This synthesized resin is selective for the nickel and cobalt ions which are strongly bound to the chelating resin.

Studies of ion-exchange kinetics fall into three broad classifications. The following general mechanisms were shown to dominate the overall rate: 1) the diffusion of ions in the external solution is usually termed film diffusion, 2) the diffusion of ions within the exchange is termed particle diffusion, and 3) the exchange of ions at the sites of the functional groups of the exchanger, which is termed chemical exchange, may be the slowest step depending upon the nature of the complexes formed (14).

The rate-controlling step at the beginning of the reaction was found to depend upon how tightly the ligand binds to the resin, so the actual chemical-exchange reaction is the rate-determining step (15). The actual chemical-exchange reaction is unaffected by the stirring rate. The particle diameter of the resin (32 μm) did not change during the exchange process. The elucidation of the rate-determining step of the reaction rate proves that the reaction rate is pseudo-first order with respect to the concentration of the active H^+ points on the resin phase (16).

In the kinetics part, rate measurement has been carried out by measuring the pH of the reaction mixture (Table 2). The immobilized metal ion in the resin matrix was cobalt, which is known to give strong complexes with ligands.

Measurements of the pH of the reaction mixture were made for at least an hour. It is clear from the experimental data presented that the rate of cytidine binding is determined largely by the rate of the chemical-exchange

TABLE 2
pH Changes in Cytidine–Ligand-Exchange Reaction

t (min)	pH	a_{H^+} ($\times 10^{-5}$)
0	4.03	9.33
4	4.16	6.91
8	4.24	5.75
12	4.29	5.13
16	4.33	4.68
20	4.37	4.27
24	4.40	3.98
28	4.42	3.80
32	4.44	3.63
36	4.46	3.47
40	4.48	3.31
44	4.48	3.31

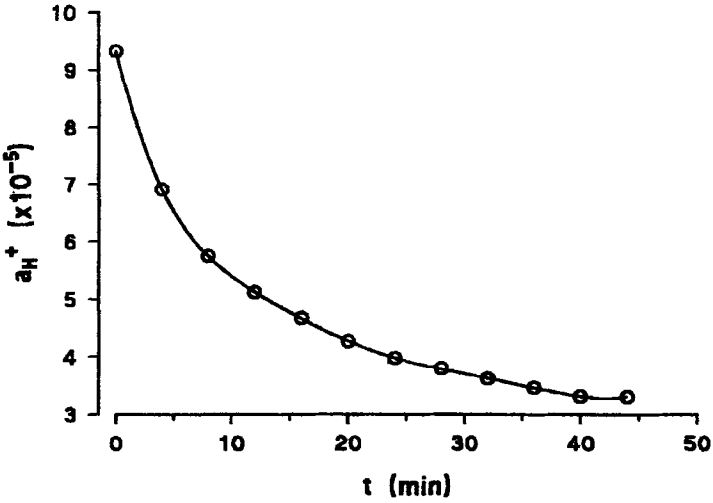


FIG. 3 Time dependence of a_{H^+}: 0.5 g resin, 25°C.

reaction between ligand and resin. Cobalt(II) cation in the resin matrix gives a chelate with cytidine during the exchange reaction. It can be seen from Fig. 3 that the first experimental points fit a straight line very well. It can be accepted that the rate at the beginning of the reaction, V_0, is the slope of the straight line, and its value was calculated to be 0.6.

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