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CHROMATOGRAPHIC SEPARATION OF PYRIDINE AND ADENINE NUCLEOTIDES ON THIN LAYERS OF POLY(ETHYLENEIMINE) CELLULOSE

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SUMMARY

The chromatographic properties on thin layers of poly(ethyleneimine) cellulose of sixteen compounds containing the pyridine and/or adenine ring have been studied. Chromatographic mobilities have been examined as a function of the concentration of lithium chloride or sodium formate buffer in the chromatographic solvent. These data provide a rationale for the development of rapid and simple separation methods that should prove useful in the study of pyridine and adenine nucleotide metabolism.

INTRODUCTION

Anion-exchange chromatography has proven to be a valuable method for the separation of nucleotides. Two of the most widely used systems for the separation of complex mixtures of nucleotides have been liquid chromatography on columns of anion-exchange resins[1–5] and thin-layer chromatography (TLC) on poly(ethyleneimine) cellulose[6,7]. The resolution of a large number of pyridine- and/or adenine-containing compounds by liquid chromatography on Dowex 1-formate has been reported[8–10]. These methods offer excellent resolution but are time consuming because they require elution with a complex gradient of large volume or with stepwise concentrations of eluents. Anion-exchange TLC offers high resolution and has the combined advantages of speed and simplicity[6,7]. Studies on pyridine nucleotide metabolism would be facilitated by the availability of methods that would allow the rapid separation of nucleotides that contain the pyridine and/or adenine ring. The chromatographic properties on PEI-cellulose thin layers of several adenine nucleotides and the two major pyridine nucleotides NAD^+ and $NADP^+$ have been studied[6]. We report here a systematic study of the chromatographic properties of sixteen compounds containing the pyridine and/or adenine ring on thin layers of PEI-cellulose. These studies have allowed the development of rapid and simple methods for the simultaneous separation of a large number of intermediates of pyridine and adenine nucleotide metabolism.

EXPERIMENTAL*

Pyridine and adenine compounds

Adenine, adenosine, nicotinamide, nicotinic acid, NMN, NAD^+, $NADP^+$, AMP, cAMP, ADPR, ADP, and ATP were obtained from Sigma (St. Louis, Mo., U.S.A.).

$NaAD^+$ was prepared as described by Honjo and Nishizuka[11], except that commercial pig brain NAD glycohydrolase (Sigma, Cat. No. N9879) was used instead of beef spleen NAD glycohydrolase. The 100-ml incubation contained 17.5 mg/ml of the NAD glycohydrolase preparation and incubation was for 50 min.

NaMN was prepared by incubation of $NaAD^+$ with snake venom phosphodiesterase (Worthington, Freehold, N.J., U.S.A. Cat. No. VPH 3926). The incubation mixture (1.35 ml) contained 74 m*M* Tris–HCl of pH 8.5, 37 m*M* $MgCl_2$, 0.84 m*M* $NaAD^+$, and 20 units (0.68 mg) of snake venom phosphodiesterase. After incubation at 30° for 30 min, the mixture was placed in a boiling water-bath for 1 min and then chilled in an ice-bath. The mixture was centrifuged and the supernatant was applied to a 15 × 0.9 cm I.D. column of Bio-Rad AG 1-X4 (200–400 mesh) in the formate form. The column was eluted with a 500-ml linear gradient of water to 2.0 *N* formic acid. The order of elution from the column is AMP, NaMN, and unreacted $NaAD^+$. The fractions containing NaMN were lypholyzed, resuspended in water, and frozen.

Nicotinamide ribonucleoside and nicotinic acid ribonucleoside were prepared by quantitative conversion of the corresponding nucleotide to nucleoside in the presence of alkaline phosphatase. The incubation mixture (1 ml) for nicotinamide ribonucleoside contained 100 m*M* Tris–HCl of pH 8.5, 10 m*M* NMN, and 0.35 mg of *Escherichia coli* alkaline phosphatase (Sigma, Cat. No. P4252). After incubation at 37° for 30 min, the mixture was boiled for 1 min and cooled in an ice-bath for 5 min. Denatured protein was removed by centrifugation, and the supernatant was frozen. The incubation mixture for nicotinic acid ribonucleoside (1 ml) contained 100 m*M* Tris–HCl of pH 8.5, 3 m*M* NaMN, and 0.35 mg of alkaline phosphatase. After incubation at 37° for 60 min, the mixture was treated as described above.

Stock solutions used for chromatography were stored as frozen aqueous solutions of 5–10 m*M*.

Thin-layer plates and chromatographic procedures

Pre-coated thin layers (0.1 mm thickness, 20 cm × 20 cm) of PEI-cellulose on plastic sheets were obtained from Brinkmann (Westbury, N.Y., U.S.A.). For prewashing, 15-cm wicks of Whatman 3MM filter paper were attached to the plates, which were developed up to 5 cm in 10% (w/v) NaCl. They were then transferred without immediate drying to distilled water and allowed to migrate to the end of the wick. After drying, the plates were eluted a second time with distilled water and dried. The wicks were removed and the plates were stored at −15° until used.

Chromatographic stock solutions of 2 *M* LiCl and 2 *M* sodium formate buffer of pH 3.6, were used to prepare chromatographic solvents. The formate stock

* The following nonstandard abbreviations are used: PEI-cellulose = poly(ethyleneimine) cellulose; ADPR = adenosine diphosphoribose; NMN = nicotinamide mononucleotide; $NaAD^+$ = nicotinic acid adenine dinucleotide, oxidized form; NaMN = nicotinic acid mononucleotide.

solution was prepared by mixing equal volumes of 2 *M* formic acid and 2 *M* sodium formate. For chromatography, 20–100 nmoles of compound were spotted with a micropipette 3 cm from the bottom edge of the plate. For two-dimensional separations, the plates were treated with anhydrous methanol following chromatography in the first dimension. After drying, a 20 cm × 20 cm plate was immersed in 200 ml of anhydrous methanol for 5 min followed by a second treatment for 5 min in 100 ml of methanol. The plate was then dried and developed in the second dimension.

RESULTS AND DISCUSSION

The chromatographic mobilities on PEI-cellulose of sixteen compounds containing the pyridine and/or adenine ring were studied as a function of the concentration of LiCl or sodium formate buffer in the chromatographic solvent. A number of these including AMP, ADP, ATP, NAD^+, and $NADP^+$ have been previously characterized[6]. These have been included in the present study in order to allow the mobilities of all sixteen compounds to be examined under a single set of conditions.

With the exception of nicotinic acid, all of the nonphosphorylated derivatives examined have chromatographic mobilities that are independent of the concentration of LiCl or sodium formate buffer in the chromatographic solvent. The chromatographic mobilities of the bases and nucleosides examined are shown in Table I.

TABLE I

R_F VALUES OF NONPHOSPHORYLATED PYRIDINE AND ADENINE DERIVATIVES ON PEI-CELLULOSE

Standard deviations are shown. The number of separate chromatographic determinations is shown in parentheses.

Compound	*Elution solvent*	
	Formate	*LiCl*
Nicotinamide	0.82 ± 0.03 ($n = 10$)	0.77 ± 0.02 ($n = 8$)
Nicotinamide ribonucleoside	0.86 ± 0.04 ($n = 10$)	0.88 ± 0.05 ($n = 8$)
Nicotinic acid ribonucleoside	0.79 ± 0.04 ($n = 10$)	0.85 ± 0.04 ($n = 3$)
Adenine	0.70 ± 0.05 ($n = 5$)	0.41 ± 0.09 ($n = 5$)
Adenosine	0.74 ± 0.08 ($n = 5$)	0.58 ± 0.04 ($n = 5$)

The data of Tables II and III show the chromatographic mobilities of nicotinic acid and ten phosphorylated compounds of pyridine and adenine nucleotide metabolism as a function of the concentration of LiCl and sodium formate buffer, respectively, in the chromatographic solvent. The R_F values shown represent the average of a minimum of three separate chromatographic runs. Absolute R_F values differ by less than ±0.05 from run to run. Of the compounds shown, only NMN and nicotinic acid show appreciable migration when eluted with water. R_F values in water of 0.46 for NMN and 0.18 for nicotinic acid were observed.

TABLE II

R_F VALUES ON PEI-CELLULOSE OF PYRIDINE AND ADENINE NUCLEOTIDES IN LiCl

Compound	*LiCl concentration*				
	0.2 M	*0.6 M*	*1.0 M*	*1.6 M*	*2.0 M*
Nicotinic acid	0.52	0.75	0.78	0.84	0.84
NMN	0.62	0.78	0.86	0.93	0.93
NAD^+	0.56	0.79	0.82	0.86	0.85
AMP	0.09	0.27	0.46	0.64	0.71
cAMP	0.39	0.58	0.57	0.62	0.61
NaMN	0.25	0.57	0.72	0.94	0.92
$NaAD^+$	0.17	0.66	0.76	0.88	0.87
ADPR	0.04	0.28	0.47	0.68	0.70
$NADP^+$	0.04	0.20	0.50	0.81	0.87
ADP	0.01	0.07	0.20	0.51	0.65
ATP	0.00	0.03	0.06	0.27	0.52

The data of Tables I, II, and III provide the necessary information for the development of chromatographic systems that allow the separation of many compounds of pyridine and adenine nucleotide metabolism. Although many of the compounds can be separated by a one-dimensional development in one of the chromatographic solvents, the LiCl and formate systems complement one another. For example, although AMP, cAMP, and NaMN are poorly resolved at all concentrations of formate buffer, they can be resolved by chromatography in 0.2 *M* LiCl. $NADP^+$ and ADPR are likewise not separated in formate buffer but can be separated by 1.6 *M* or 2 *M* LiCl. The nicotinic acid-containing nucleotides are not resolved in LiCl but can be separated by 0.25 or 0.5 *M* formate buffer. An example of the utility of these methods is shown in Fig. 1, which depicts a rapid and simple chromatographic separation of nine phosphorylated intermediates of pyridine and adenine nucleotide metabolism. Although ADPR should also be separated from the other

TABLE III

R_F VALUES ON PEI-CELLULOSE OF PYRIDINE AND ADENINE NUCLEOTIDES IN SODIUM FORMATE BUFFER

Compound	*Formate concentration*				
	0.25 M	*0.5 M*	*1.0 M*	*1.5 M*	*2.0 M*
Nicotinic acid	0.61	0.76	0.84	0.87	0.85
NMN	0.72	0.85	0.90	0.91	0.95
NAD^+	0.57	0.78	0.81	0.85	0.87
AMP	0.53	0.67	0.76	0.80	0.77
cAMP	0.53	0.63	0.72	0.75	0.79
NaMN	0.41	0.61	0.74	0.83	0.88
$NaAD^+$	0.14	0.31	0.66	0.77	0.85
ADPR	0.05	0.14	0.43	0.63	0.79
$NADP^+$	0.04	0.12	0.40	0.60	0.76
ADP	0.01	0.02	0.09	0.17	0.28
ATP	0.00	0.00	0.01	0.00	0.04

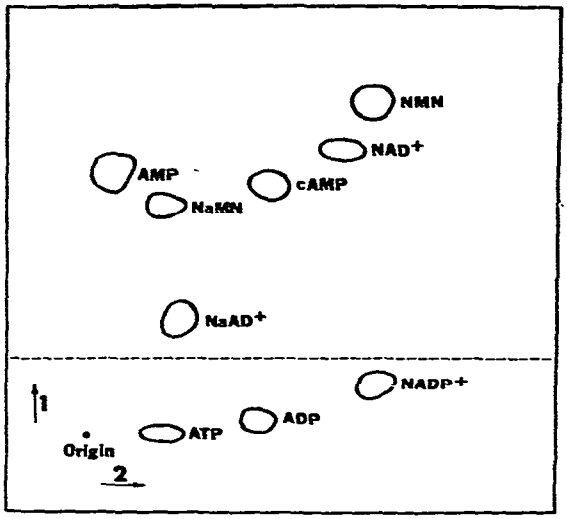

Fig. 1. Two-dimensional thin-layer chromatography of nine pyridine- and/or adenine-containing nucleotides on PEI-cellulose. The TLC plate was developed in the first direction with 0.5 *M* sodium formate buffer and was treated with anhydrous methanol as described in the text. It was then cut horizontally at 6.2 cm from the Origin, as indicated by the dashed line. The two resulting sections were developed in the second direction with 0.2 *M* LiCl for the upper section and 1.0 *M* LiCl for the section containing the origin.

nucleotides, we have observed that it does not migrate in the second dimension but instead forms a yellow spot at the location of its migration in the first dimension.

The chromatographic properties of many pyridine- and adenine-containing nucleotides by liquid chromatography on columns of Dowex 1-formate have been characterized[8–10]. The data presented here allow the application of PEI-cellulose TLC methods to achieve separation of pyridine and adenine nucleotides. PEI-cellulose TLC has many advantages which have been discussed in detail by Randerath and Randerath[6]. Among these are high resolution, speed, simplicity, and ease of quantification and/or rechromatography.

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