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RESOLUTION OF COMPLEX MIXTURES OF NUCLEIC ACID BASES, NUCLEOSIDES, AND NUCLEOTIDES BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY ON POLYETHYLENEIMINE–CELLULOSE*

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SUMMARY

A method is given for resolving a complex mixture of nucleic acid bases, nucleosides, and nucleotides on polyethyleneimine–cellulose MN-300 chromatofilms. The nucleotides are prevented from migrating when the individual nucleic acid bases and nucleosides are separated from each other. Also, in the course of the development that resolves the nucleotides, the nucleic acid bases and nucleosides disappear. Thus, the procedure simplifies the isolation and detection of individual components of a complex mixture of nucleic acid derivatives. The method was applied satisfactorily to the analysis of alkali hydrolysates of the transfer ribonucleic acids of *E. coli* B and of yeast.

INTRODUCTION

The development and applications of polyethyleneimine (PEI)–cellulose for anion-exchange thin-layer chromatography are discussed in several articles by the RANDERATHS[1–5]. The layers were supported on either glass plates or sheets of vinyl plastic. On these layers they separated mixtures of nucleotides by both one-[1] and two-dimensional[2] stepwise development with different solvents. In a recent further use of the layers, PATAKI AND NIEDERWIESER[6] resolved the components of groups of nucleic acid bases, nucleosides, and nucleotides; all the components of each of the groups had a common nucleobase. The chromatofilms were developed by gradient elution in one dimension.

The present communication describes the resolution of complex mixtures of nucleic acid bases, nucleosides, and nucleotides irrespective of their nucleobase moiety or chain length. The separations are achieved on two PEI–cellulose MN-300 chromatofilms by two-dimensional stepwise development. On one chromatofilm, the individual nucleic acid bases and nucleosides are resolved. The chromatofilm is developed with water in the first direction and with the mixed solvent *n*-butanol–methanol–water–

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ammonium hydroxide in the second dimension. The nucleotides do not migrate in either of these solvents. Their restriction to the area of the origin thus simplifies the identification of the resolved nucleic acid bases and nucleosides. On a second chromatofilm, the individual nucleotides are resolved. The chromatofilm is developed stepwise in the first dimension with 0.2, 1.0, and 1.6 *M* lithium chloride solutions and in the second dimension with 0.5, 2.0, and 4.0 *M* formic acid–sodium formate buffer solutions of pH 3.4. Between the developments in the first and second dimensions, the chromatofilm is washed with methanol to remove the lithium chloride. The methanol wash and the subsequent development with the buffer solutions cause the nucleic acid bases and the nucleosides, with one exception, to disappear completely. Thus, the nucleotides are resolved without interference from nucleic acid bases and nucleosides.

The procedure for the resolution of the nucleobases and nucleosides is based partly on that described by K. RANDERATH[7] for the two-dimensional separation of nucleic acid bases on layers of cellulose MN-300. The nucleotide separation procedure is that of E. RANDERATH AND K. RANDERATH[2].

EXPERIMENTAL

Reagents

Polyethyleneimine, 50 % aqueous solution. From Chemirad Corp. (East Brunswick, N.J.).

Cellulose MN-300 powder. Manufactured by Macherey, Nagel & Co. (Düren, Germany) and distributed by Brinkmann Instruments Inc. (Westbury, N.Y.).

Ribonucleic acid bases. Adenine, cytosine, guanine, hypoxanthine, uracil, and xanthine; from P-L Biochemicals, Inc. (Milwaukee, Wisc.). Thymine; from Mann Research Laboratories, Inc. (New York, N.Y.). 5-Methylcytosine and 5-hydroxymethylcytosine; from Sigma Chemical Co. (St. Louis, Mo.). 3-Methyluracil; from K & K Laboratories, Inc. (Plainview, N.Y.).

Ribonucleosides. Adenosine, cytidine, guanosine, inosine, thymidine, and uridine; from Schwarz BioResearch, Inc. (Orangeburg, N.Y.). Xanthosine; from P-L Biochemicals, Inc. 1-Methyladenosine; from Sigma Chemical Co. 1-Methylguanosine and 1-methylinosine; from Cyclo Chemical Corp. (Los Angeles, Calif.).

*Ribonucleotides**. 2′,3′-AMP, 2′,3′-CMP, 2′,3′-GMP, and 2′,3′-UMP; from Mann Research Laboratories, Inc. 5′-CMP, 5′-GMP, 5′-UMP, and 5′-XMP; from P-L Biochemicals, Inc. 5′-AMP, 5′-IMP, 5′-ATP, and 5′-CTP; from Sigma Chemical Co.

Apparatus

Adsorbent applicator. The adsorbent applicator was the Desaga type, which is available from Brinkmann Instruments Inc. Instead of the plastic template, a glass template was used according to the instructions of K. RANDERATH AND E. RANDERATH[5].

Viewing cabinet. The chromatograms were viewed in a Spectroline C-3F viewing cabinet, available from Black Light Eastern, Spectronics Corp. (Westbury, Long Island, N.Y.).

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* AMP, CMP, GMP, IMP, UMP, XMP = monophosphates of adenosine, cytidine, guanosine, inosine, uridine, xanthosine; ATP, CTP = corresponding triphosphates.

Procedures

Preparation of chromatofilms. The PEI–cellulose MN-300 chromatofilms of 500-μ layer thickness were prepared on rigid vinyl sheets as instructed by K. RANDERATH AND E. RANDERATH[5]. Impurities were removed from the chromatofilms according to Procedure 3 of K. RANDERATH AND E. RANDERATH[5]. The ascending irrigation with distilled water was carried out only until the water front had just reached the top edge of the layer; a longer irrigation period caused the water-soluble PEI to be removed from the lower area of the chromatofilms.

Spotting. The test solutions were deposited on the chromatofilms in 1-μl portions. To increase the total amount of material deposited on a chromatofilm, replicate portions were deposited on the same spot, the portions being dried at room temperature after each deposition.

Development of chromatofilms. The chromatofilms were developed by upward migration of the solvent in chromatographic jars (7 1/8 in. wide by 9 3/8 in. long by 12 3/4 in. deep) covered with plastic film (Saran Wrap). Test portions were deposited at a point 3 cm from the bottom and 3 cm from the left side of the chromatofilm. The chromatofilms were immersed in the solvent to a depth of about 1.5 cm.

For the separation of the individual nucleic acid bases and nucleosides, the chromatofilms were developed for a distance of 10 cm from the origin with triple-distilled water. They were then dried in air. The dry chromatofilms were developed in the second direction with the mixed solvent *n*-butanol–methanol–water–conc. ammonium hydroxide (60:20:20:1, v/v) that was prepared freshly. They were dried in air and then viewed.

For resolving the nucleotides, the chromatofilms were developed in the first dimension for 2 min with 0.2 *M* LiCl, for 6 min with 1.0 *M* LiCl, and to a distance 13 cm from the origin with 1.6 *M* LiCl. The chromatofilms were dried and then were washed for 15 min in 1 l of absolute methanol. They were drained and dried thoroughly in air. They were developed stepwise in the second dimension with formic acid–sodium formate buffer solutions of pH 3.4 as follows: 30 sec with 0.5 *M* buffer, 2 min with 2.0 *M* buffer, and to a distance 15 cm from the origin with 4.0 *M* buffer. The chromatofilms were dried in air and viewed.

Detection of the resolved constituents. The positions of the resolved constituents on the chromatograms were determined by observing the chromatograms in a viewing cabinet under light of 254 mμ wavelength. The resolved constituents appeared as dark (violet) spots against the slightly fluorescing yellow background of the chromatofilms.

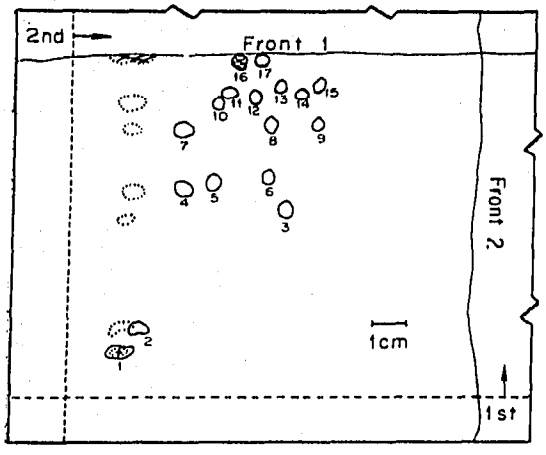
RESULTS AND DISCUSSION

To evaluate the separation procedure, a solution was chromatographed that contained the ten nucleic acid bases, ten nucleosides, and twelve nucleotides that are listed under Reagents. The solution was about 0.33 m*M* in each component except those that were insufficiently soluble, in which cases the solution was saturated with them.

Isolation of individual nucleic acid bases and nucleosides

The efficiency of the procedure for isolating individual nucleic acid bases and nucleosides is shown by Fig. 1. Of the twenty nucleobases and nucleosides in the test

solution, only three appeared in the same spot together with another component: cytosine with xanthine, uridine with cytidine, and 1-methylinosine with 5-methylcytosine. The violet fluorescence of 1-methylguanosine distinguishes it from the other components. The twelve nucleotides remain together at the original spot and thus do not interfere with the identification of the nucleobases and nucleosides.



COMPONENTS

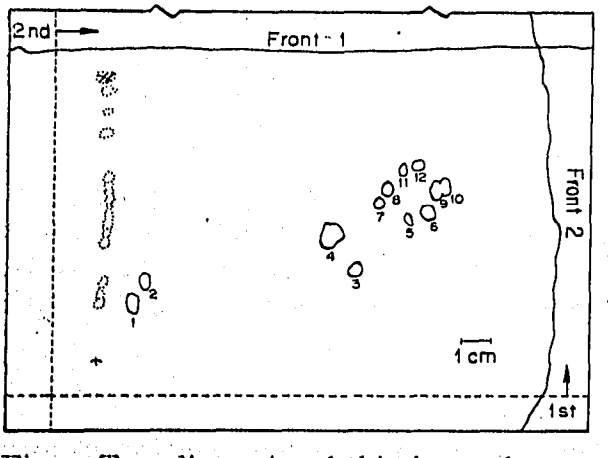
1. Nucleotides
2. Xanthosine
3. Adenine
4. Guanosine
5. Hypoxanthine
6. Adenosine
7. Inosine
8. Uracil
9. Thymine
10. Cytosine + xanthine
11. Uridine + cytidine
12. 5-Methylcytosine + 1-methylinosine
13. Guanine (?)
14. 3-Methyluracil
15. Thymidine
16. 1-Methylguanosine
17. 1-Methyladenosine

+ = Origin; ◌ = position after first development;
○ = position after second development; ⊗ = violet fluorescence

Fig. 1. Two-dimensional thin-layer chromatogram of nucleic acid bases and nucleosides isolated from a complex mixture of nucleic acid bases, nucleosides, and nucleotides. (– – – –) = Immersion line. Test portion: 1 μl × 9 = 9 μl. See text for other details.

Isolation of individual nucleotides

The excellent resolution achieved for the nucleotides is shown by Fig. 2. Of the twelve nucleotides present in the mixture chromatographed, only 2′,3′-GMP and 5′-GMP are unresolved; only partial resolution is achieved for 2′,3′-CMP and 5′-CMP. No doubt with slight modification to the development procedure, the complete resolution of these nucleotides can be achieved. For example, Fig. 3 shows that 2′-, 3′-, and 5′-AMP can be completely resolved if the time for development with 0.5 *M* buffer is increased from 30 to 45 sec.



COMPONENTS

1. 5′-ATP
2. 5′-CTP
3. 5′-XMP
4. 2′,3′-GMP + 5′-GMP
5. 2′,3′-AMP
6. 5′-AMP
7. Xanthosine
8. 5′-IMP
9. 2′,3′-CMP
10. 5′-CMP
11. 2′,3′-UMP
12. 5′-UMP

Fig. 2. Two-dimensional thin-layer chromatogram of nucleotides isolated from a complex mixture of nucleic acid bases, nucleosides, and nucleotides. See Fig. 1 and text for other details.

The nucleobases and nucleosides (except xanthosine) disappear completely during the methanol wash and the development in the second direction. Xanthosine migrates and is resolved but is not removed from the chromatogram; this unique behavior is a means for identifying xanthosine. The disappearance of these components possibly results from their solubility in methanol.

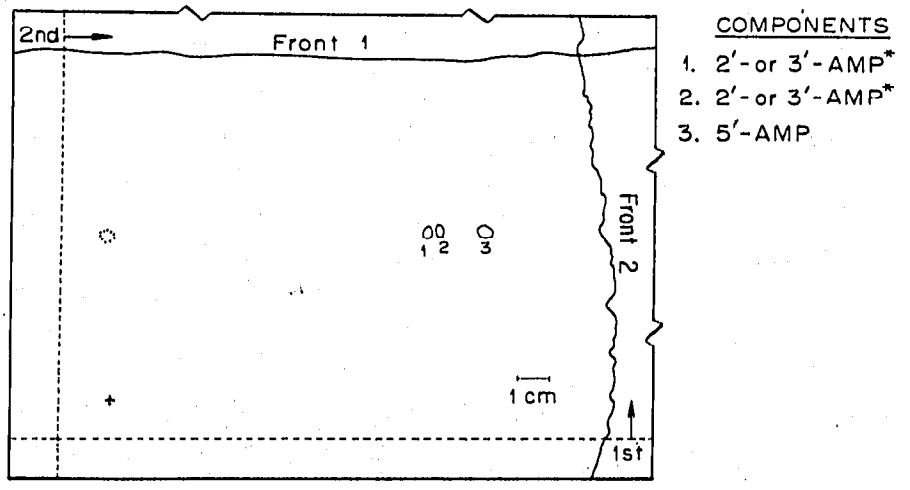


Fig. 3. Two-dimensional thin-layer chromatogram of 2'-, 3'-, and 5'-AMP. Test portion: 1 μl × 3 = 3 μl. Development time with 0.5 *M* buffer: 45 sec. See Fig. 1 and text for other details. The chromatogram was composed from a chromatogram of 2',3'-AMP and a chromatogram of 5'-AMP. Other experiments have shown that the positions of these resolved constituents are the same whether they are present alone or together in the test solution.

* Neither 2'-AMP nor 3'-AMP was available in pure form for use as a reference.

Reproducibility of the separations

The reproducibilities of the separations of the individual nucleobases and nucleosides and of the nucleotides are excellent. In the experiments that showed this, replicate chromatograms were run in each case on chromatofilms prepared from the same batch of PEI–cellulose MN-300, the developing solvents originally prepared were used, and the portions of test solutions were chromatographed on different days. PATAKI AND ZÜRCHER[8] also give information on the reproducibility of separations of nucleoderivatives on this type of layer.

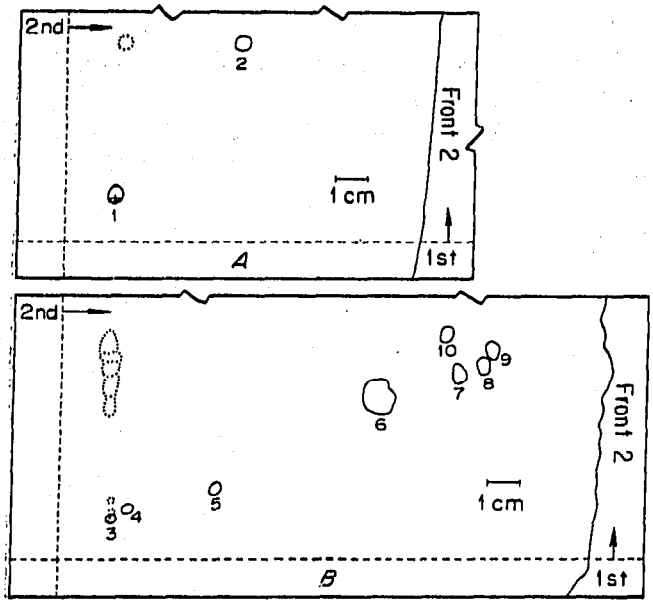
Application of the method to alkali hydrolysates of transfer ribonucleic acids

The procedure was used satisfactorily to separate the nucleic acid components of alkali hydrolysates of transfer ribonucleic acids (tRNA's) of *E. coli* B and of yeast; Figs. 4 and 5, respectively, present the results. The nucleosides were estimated to be present in about 1/70 the amounts of the nucleotides.

In these analyses it was found advantageous to use several different total test-portion volumes (*e.g.*, 3, 6, and 12 μl) in order both to detect those components present in the smaller concentrations and to resolve those present in the larger concentrations.

Evaluation of commercially available PEI–cellulose MN-300 chromatofilms

The PEI–cellulose MN-300 chromatofilms available from one commercial source were compared with freshly prepared chromatofilms of the same type for the

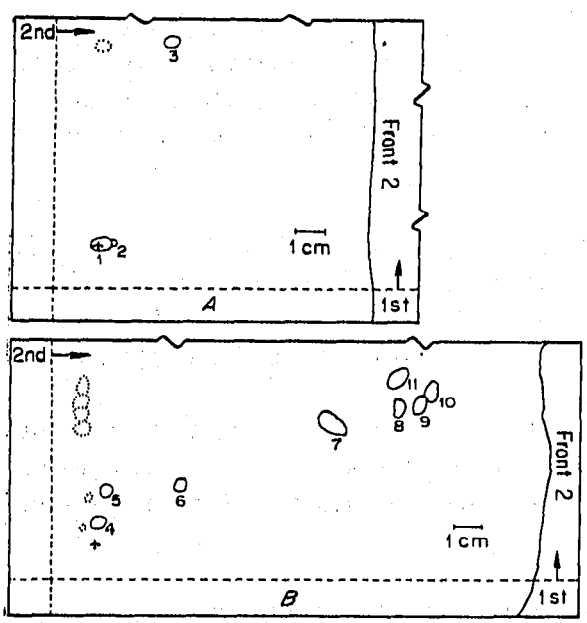


COMPONENTS

1. Nucleotides
2. Adenosine
3. ?
4. ?
5. A diphosphate
6. 2', 3'-GMP
7. 2'- or 3'-AMP*
8. 2'- or 3'-AMP*
9. 2', 3'-CMP
10. 2', 3'-UMP

Fig. 4. Two-dimensional thin-layer chromatograms of an alkali hydrolysate of tRNA of *E. coli* B. Chromatogram A, isolation of nucleobases and nucleosides; chromatogram B, isolation of nucleotides. Test portion: 1 μl × 3 = 3 μl in each case. See Fig. 1 and text for other details.

* Neither 2'-AMP nor 3'-AMP was available in pure form for use as a reference.



COMPONENTS

1. Nucleotides
2. ?
3. Cytidine
4. ?
5. ?
6. A diphosphate
7. 2', 3'-GMP
8. 2'- or 3'-AMP*
9. 2'- or 3'-AMP*
10. 2', 3'-CMP
11. 2', 3'-UMP

Fig. 5. Two-dimensional thin-layer chromatograms of an alkali hydrolysate of tRNA of yeast. Test portion: 1 μl × 6 = 6 μl. See Fig. 4 and text for other details.

thin-layer chromatography of the nucleic acid components according to this method. These commercial films have several disadvantages. They are provided in one thickness only (100 μ). When used under the same conditions as the freshly prepared 500-μ-thick layers, they require longer development time, give spots of larger area (and therefore have poorer resolution), and show pronounced tailing. These differences may result from their being thinner. They are less sensitive. When they are used directly as received and are developed with water, they form a finger-like deposit of yellow fluorescent PEI, which is water soluble, for a distance of as much as about 4 cm behind the solvent front. This deposit distorts and obscures the spots of substances that migrate near the solvent front. If the commercial films are pretreated according to the procedure used in processing the freshly prepared chromatofilms, this effect is eliminated, but the pretreated films still have the other disadvantages. PATAKI AND ZÜRCHER[8] also have compared their freshly prepared PEI–cellulose MN-300 chromatofilms with the same type of chromatofilms available from two commercial sources; the compounds separated in the comparison were also nucleoderivatives.

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